

Inhibitory G proteins play multiple roles to polarize sensory hair cell morphogenesis

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Abstract

Inhibitory G alpha (GNAI or Gai) proteins are critical for the polarized morphogenesis of sensory hair cells and for hearing. The extent and nature of their actual contributions remains unclear, however, as previous studies did not investigate all GNAI proteins and included non-physiological approaches. Pertussis toxin can downregulate functionally redundant GNAI1, GNAI2, GNAI3 and GNAO proteins, but may also induce unrelated defects. Here we directly and systematically determined the role(s) of each individual GNAI protein in mouse auditory hair cells. GNAI2 and GNAI3 are similarly polarized at the hair cell apex with their binding partner GPSM2, whereas GNAI1 and GNAO are neither detected nor polarized. In *Gnai3* mutants, GNAI2 progressively fails to fully occupy the subcellular compartments where GNAI3 is missing. In contrast, GNAI3 can fully compensate for the loss of GNAI2 and is essential for hair bundle morphogenesis and auditory function. Simultaneous inactivation of *Gnai2* and *Gnai3* recapitulates for the first time two distinct types of defects only observed so far with pertussis toxin: 1) a delay or failure of the basal body to migrate off-center in prospective hair cells, and 2) a reversal in the orientation of some hair cell types. We conclude that GNAI proteins are critical for hair cells to break planar symmetry and to orient properly before GNAI2/3 regulate hair bundle morphogenesis with GPSM2.

eLife assessment

This **important** study provides insights into the functions of different GNAI subunits, substantially advancing our understanding of their roles in the regulation of hair cell stereociliary bundle development. **Convincing** evidence is provided to support the findings and overall, the results of this study provide a thorough and careful examination of the roles of different GNAIs in the inner ear, with only minor weaknesses identified in review.

Developing sensory hair cells (HCs) in the inner ear undergo a complex polarization process to detect and interpret mechanical stimuli, including sound. Each mature HC is able to detect stimuli in a directional manner by developing an asymmetric brush of actin-based membrane protrusions, or stereocilia: the hair bundle. Neighboring HCs also adopt concerted orientations to align their hair bundles, a property known as planar cell polarity (PCP). One subclass of heterotrimeric guanine nucleotide-binding (G) protein was intimately associated with different levels of mouse HC polarization: inhibitory G alpha subunits (GNAI1, GNAI2, GNAI3 and GNAO; collectively GNAI or Gai). However, several roles proposed for GNAI proteins have not been validated physiologically, and the individual contribution of each GNAI remains uncertain.

HC polarization along the epithelial plane starts with the off-center migration of the basal body and its associated primary cilium, the kinocilium (Denman-Johnson & Forge, 1999 [↗](#); Mbiene & Sans, 1986 [↗](#); Tilney et al, 1992 [↗](#)). At that stage, Regulator of G protein Signaling 12 (RGS12) is required for GNAI and the G protein signaling modulator 2 (GPSM2) scaffold to form a polarized complex at the apical membrane on the side of the off-center basal body (Akturk et al, 2022 [↗](#); Ezan et al, 2013 [↗](#); Tarchini et al, 2013 [↗](#)). GPSM2-GNAI is best known as a highly conserved protein complex orienting the mitotic spindle during progenitor divisions (Du et al, 2001 [↗](#); Schaefer et al, 2001 [↗](#); Schaefer et al, 2000 [↗](#); Woodard et al, 2010 [↗](#)). In post-mitotic HCs, GPSM2-GNAI first excludes microvilli and microvilli-derived stereocilia from the portion of the HC surface where it resides, the expanding bare zone (**Figure 1A** [↗](#)). Bare zone expansion then pushes back the basal body/kinocilium from a more eccentric position near the lateral junction to a less eccentric position at the vertex of the forming hair bundle. Later, GPSM2-GNAI becomes enriched at the distal tip of row 1 stereocilia abutting the bare zone (Tarchini et al, 2016 [↗](#)). In these stereocilia, GPSM2-GNAI is a module of the elongation complex that also comprises MYO15A, WHRN and EPS8 (Mauriac et al, 2017 [↗](#); Tadenev et al, 2019 [↗](#)). GPSM2-GNAI is required at row 1 tips for boosting enrichment of other elongation complex partners, and presumably actin incorporation, compared to further stereocilia rows. GPSM2-GNAI thus confers row 1 its tallest identity and the hair bundle its asymmetric graded-height morphology.

Pertussis toxin (ptx) has been extensively used as a tool to ADP-ribosylate the GNAI subunit and dissociate heterotrimeric G protein complexes from GPCRs to inactivate downstream signaling (Locht et al, 2011 [↗](#)). *In vivo* expression of ptx catalytic subunit (ptxA) prevents normal enrichment and polarization of GPSM2-GNAI in developing HCs (Tadenev et al, 2019 [↗](#); Tarchini et al, 2013 [↗](#)), suggesting that ADP-ribosylation directly or indirectly inhibits GPSM2-GNAI function as well. Ptx recapitulates severe stereocilia stunting and immature-looking hair bundles observed when GPSM2 or both GNAI2 and GNAI3 are inactivated (Beer-Hammer et al, 2018 [↗](#); Mauriac et al, 2017 [↗](#); Tadenev et al, 2019 [↗](#); Tarchini et al, 2016 [↗](#)). In contrast, stereocilia height is more variably reduced in *Gnai3* single mutants, with defects more severe at the cochlear base (Beer-Hammer et al, 2018 [↗](#); Mauriac et al, 2017 [↗](#)). This can explain why hearing loss is more severe at high frequencies in *Gnai3* mutants, but profound at all frequencies in a *ptxA* model and in mutants lacking GPSM2 or both GNAI2 and GNAI3 (Beer-Hammer et al, 2018 [↗](#); Mauriac et al, 2017 [↗](#); Tarchini et al, 2016 [↗](#)). Surprisingly, before affecting hair bundle differentiation at postnatal stages, ptx also causes two distinct defects in HC polarization at embryonic stages.

First, one study reported that a high dose of ptx in cultured explants of the developing cochlea resulted in a low proportion of symmetrical HCs with a central kinocilium surrounded by a rounded hair bundle (**Figure 1B** [↗](#)) (Ezan et al, 2013 [↗](#)). Because GPSM2-GNAI recruits partners to pull on astral microtubules during mitotic spindle orientation, the authors proposed that GPSM2-GNAI similarly orients the migration of the basal body when post-mitotic HCs break planar symmetry. However, this hypothesis has not been validated *in vivo* to date. Studies where *Gpsm2* (Bhonker et al, 2016 [↗](#); Ezan et al, 2013 [↗](#); Tarchini et al, 2013 [↗](#)), *Gnai3* (Beer-Hammer et al,

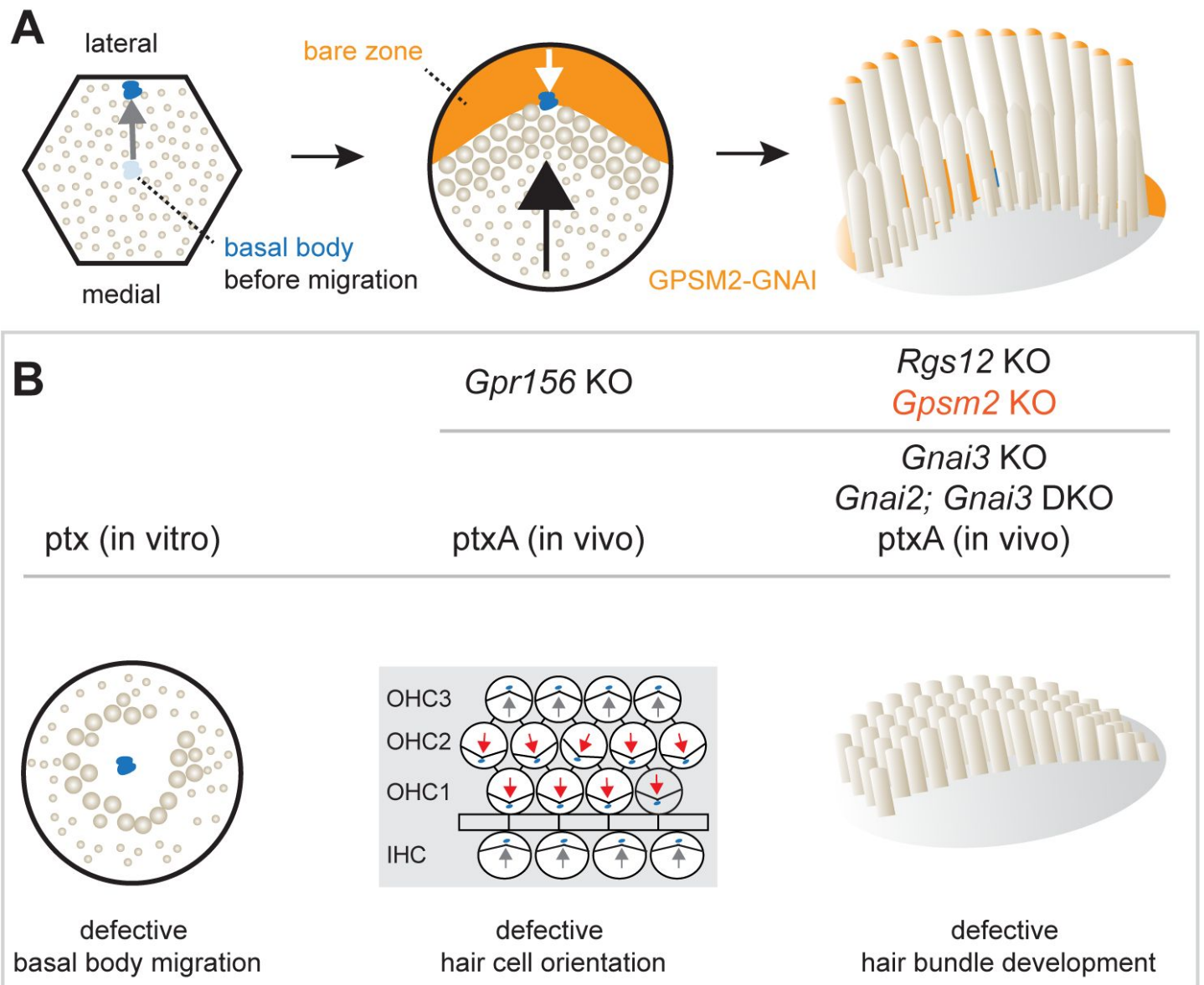


Figure 1.

Summary of GNAI-related functions proposed previously.

A) Apical HC differentiation from symmetry breaking to hair bundle development. The distribution of the GPSM2-GNAI complex at the bare zone and at stereocilia tips is indicated in orange. **B)** Defects observed with pertussis toxin (ptx) or when inactivating GNAI proteins. Defective off-center migration of the basal body and inverted OHC1-2s were only observed with ptx, respectively in cochlear explants (in vitro) and by expressing the ptx catalytic subunit (ptxA) in vivo. Mouse knock-out (KO) of *Gnai* genes were to date only reported to affect hair bundle morphogenesis. Known GNAI regulators that produce similar defects when inactivated are indicated on top for each type of defect. DKO, double KO.

2018 [\[1\]](#); Ezan et al, 2013 [\[2\]](#)) or *Gnai2*; *Gnai3* (Beer-Hammer et al, 2018 [\[3\]](#)) were inactivated did not report symmetrical HCs. In addition, mouse strains expressing *ptxA* *in vivo* also did not produce symmetrical cochlear HCs (Tarchini et al, 2013 [\[4\]](#); Tarchini et al, 2016 [\[5\]](#)).

Second, *ptx* experiments induced striking HC misorientation. In the cochlea, misorientation manifested as a 180° inversion of outer HCs in the first and second row (OHC1-2) whereas inner HCs (IHCs) and OHC3 were much less affected (**Figure 1B** [\[6\]](#)) (Ezan et al, 2013 [\[2\]](#); Kindt et al, 2021 [\[7\]](#); Tarchini et al, 2013 [\[4\]](#)). In vestibular organs, *ptxA* expression abrogated the line of polarity reversal and thus mirror-image HC organization in the maculae, the utricle and saccule (Jiang et al, 2017 [\[8\]](#); Kindt et al, 2021 [\[7\]](#)). Similar defects were observed by inactivating two endogenous mouse proteins, the transcription factor *EMX2* in the maculae (Jiang et al, 2017 [\[8\]](#)) and the orphan GPCR *GPR156* in the cochlea and the maculae (Kindt et al, 2021 [\[7\]](#)). These recent studies uncovered an *EMX2*>*GPR156*>*GNAI* signaling cascade that regionally reverses HC orientation in macular organs to produce mirror-image HC organization. *GNAI* signals downstream of *GPR156* to apparently reverse the orientation of basal body migration in *Emx2*-positive compared to *Emx2*-negative HCs (Tona & Wu, 2020 [\[9\]](#)) (reviewed in (Tarchini, 2021 [\[10\]](#))). While *ptx*-induced HC misorientation is thus a physiologically relevant phenotype, HC misorientation was not reported in single *Gnai3* or double *Gnai2*; *Gnai3* mutants (Beer-Hammer et al, 2018 [\[3\]](#); Mauriac et al, 2017 [\[11\]](#)).

In summary, the importance of the *GPSM2*-*GNAI* complex for hair bundle development is well-established, but multiple discrepancies cast a doubt on whether *GNAI* proteins also assume earlier polarization roles. Specific questions include whether *GNAI* proteins participate in the mechanism that pushes the basal body away from the cell center, and in the distinct *EMX2*>*GPR156* mechanism that makes a binary decision on the direction of this push (**Figure 1** [\[6\]](#)). Furthermore, it remains unclear whether *GNAI2* and *GNAI3* adopt similar or distinct distributions in HCs, and a potential role for *GNAI1* or *GNAO* has not been addressed.

In this study, we embarked on a systematic analysis of single and combined *Gnai1*, *Gnai2*, *Gnai3* and *Gnao* mutants to solve the actual role(s) of *GNAI* proteins during HC differentiation. Our results show that *GNAI3* is the only *GNAI/O* protein required for normal hair bundle morphogenesis and normal auditory brainstem response (ABR) thresholds. In absence of *GNAI3*, *GNAI2* can fully compensate at embryonic stages but is not enriched with *GPSM2* long enough to ensure normal hair bundle morphogenesis at postnatal stages. We also directly demonstrate that *GNAI* proteins have two polarization roles independent of *GPSM2* during embryogenesis. In sum, *GNAI* function is instrumental for HCs to a) break planar symmetry, b) adopt a proper binary orientation along the PCP axis downstream of *EMX2* and *GPR156*, and c) elongate and organize stereocilia into a functional hair bundle with *GPSM2*.

Results

A near-comprehensive collection of *Gnai/o* mouse mutants

In order to interrogate the individual and combined roles of all inhibitory G proteins during HC differentiation, we obtained or generated mouse strains to build a collection of single and double *Gnai/o* mutants. Single *Gnai1* and *Gnai3* mutants were derived from the *Gnai1*^{tm1Lbi}; *Gnai3*^{tm1Lbi} double mutant strain (hereafter *Gnai1*^{neo}; *Gnai3*^{neo}) (Jiang et al, 2002 [\[12\]](#)) by segregating individual mutations upon breeding (see Methods and **Supp. Table 1** [\[13\]](#) for details). We generated a new constitutive *Gnai2* mutant strain (*Gnai2*^{del}) carrying a deletion of exons 2 to 4 (**Figure 2** [\[14\]](#) **Supp. 1A** [\[15\]](#); see Methods). Finally, we obtained and derived two *Gnao1* mutant strains: a constitutive inactivation where a neomycin cassette disrupts exon 6 (*Gnao1*^{neo}) (Jiang et al, 1998 [\[16\]](#)) and the consortium alleles *Gnao1*^{tm1c(EUCOMM)Hmg} (conditional inactivation; hereafter *Gnao1*^{flx}). As simultaneous constitutive loss of *GNAI1* and *GNAI2* was reported as viable (Plummer et al,

2012 [\[4\]](#)), we established a *Gnai1^{neo}; Gnai2^{del}* double mutant strain in addition to *Gnai1^{neo}; Gnai3^{neo}* (Jiang et al, 2002 [\[4\]](#)). In contrast, double inactivation of *Gnai2; Gnai3* is lethal around Embryonic (E) day 10.5, before HCs are born (Gohla et al, 2007 [\[4\]](#)). Consequently, we generated a new *Gnai3^{fllox}* strain by flanking exons 2 and 3 with *loxP* sites (**Figure 2 Supp.1B** [\[4\]](#); see Methods). We then generated conditional *FoxG1-Cre; Gnai2^{del}; Gnai3^{fllox}* double mutants where *Gnai3* inactivation occurs as early as E8.5 in the otic vesicle (Hebert & McConnell, 2000 [\[4\]](#)). Investigating all *Gnai/o* strains in the same genetic background was unrealistic for feasibility and lethality reasons. We reasoned that the key steps in HC apical differentiation we study are probably hardwired and less likely to be influenced by genetic heterogeneity compared to susceptibility to disease, for example.

As Pertussis toxin produced two of the three types of defects associated with GNAI/O function (**Figure 1B** [\[4\]](#)), *Gnai/o* strains above needed to be compared to a strain expressing ptxA in HCs. We used our *R26^{LSL-myc:ptxA}* strain (hereafter *LSL-myc:ptxA*) expressing N-terminal myc-tagged ptxA after Cre-recombination (Tarchini et al, 2016 [\[4\]](#)). Because a related *R26^{LSL-ptxA:myc}* strain carrying a C-terminal myc tag (Regard et al, 2007 [\[4\]](#)) caused much milder HC misorientation than *LSL-myc:ptxA* in the vestibular system (Jiang et al, 2017 [\[4\]](#); Kindt et al, 2021 [\[4\]](#)), we questioned whether the myc tag could weaken toxin activity even perhaps when located N-terminal. Consequently, we generated a new strain, *R26^{DIO-ptxA}* (hereafter *DIO-ptxA*), where untagged ptxA is flanked by double-inverted *lox* sites and flipped from the non-coding to the coding strand upon Cre recombination (**Figure 2 Supp. 1C-D** [\[4\]](#); see Methods) (Schnutgen et al, 2003 [\[4\]](#)). In *DIO-ptxA*, ptxA expression is driven by a strong artificial CAG promoter, and not by the endogenous *R26* promoter as in previous strains (Regard et al, 2007 [\[4\]](#); Tarchini et al, 2016 [\[4\]](#)). We bred *LSL-myc:ptxA* and *DIO-ptxA* either with *FoxG1-Cre* (Hebert & McConnell, 2000 [\[4\]](#)), or with *Atoh1-Cre* (Matei et al, 2005 [\[4\]](#)) to limit GNAI/O inhibition to post-mitotic HCs. **Supplementary Table 1** [\[4\]](#) summarizes the strains used in this study, their origin, genetic background and viability.

Only GNAI3 is required for hair bundle morphogenesis yet other GNAI proteins participate

To investigate the role of individual GNAI/O proteins in HC development, we imaged hair bundles in 3 week-old animals at mid-cochlear position using scanning electron microscopy. In *Gnai3^{neo}* mutants, some OHC hair bundles appeared truncated, and IHCs displayed variably shortened row 1 stereocilia as well as supernumerary stereocilia rows (**Figure 2A-C** [\[4\]](#)), as previously described (Mauriac et al, 2017 [\[4\]](#)). Single inactivation of *Gnai1* or *Gnai2*, and double *Gnai1; Gnai2* inactivation did not produce overt defects, yet quantifications revealed a subtle reduction in row 1 height in all cases (**Figure 2A** [\[4\]](#), C). Accordingly, *Gnai1^{neo}; Gnai3^{neo}* double mutants appeared more severely affected than single *Gnai3^{neo}* mutants: IHC row 1 height was further reduced (from ~32% to ~47% reduced compared to littermate controls; **Figure 2C** [\[4\]](#)), and the number and width of row 1 stereocilia were respectively only significantly increased or decreased in *Gnai1^{neo}; Gnai3^{neo}* mutants (**Figure 2D-E** [\[4\]](#)). In contrast, a constitutive (*Gnao1^{neo}*) or conditional (*Atoh1-Cre; Gnao1^{fllox}*) inactivation of GNAO did not produce overt apical HC defects, and conditional inactivation of GNAO did not enhance defects in the *Gnai1^{neo}; Gnai3^{neo}* background (**Figure 2 Supp. 1E-F** [\[4\]](#)).

As reported previously, *myc:ptxA* expression in post-mitotic HCs did not only stunt stereocilia and produce extra rows, as seen in *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutants (**Figure 2A-F** [\[4\]](#)), but also inverted OHC1-2 (**Figure 2A** [\[4\]](#)) (Kindt et al, 2021 [\[4\]](#); Tarchini et al, 2013 [\[4\]](#); Tarchini et al, 2016 [\[4\]](#)). This defect was proposed to reflect an earlier role for GNAI proteins downstream of the GPR156 receptor to define the binary orientation of HCs along the PCP axis (Kindt et al, 2021 [\[4\]](#)). Despite breeding for over two years, we could only obtain one single *Gnai2^{neo}; Gnai3^{fllox}* adult double mutant due to postnatal lethality (*FoxG1-Cre; Gnai2^{del/del}; Gnai3^{fllox/fllox}*; see **Supp. Table 1** [\[4\]](#)). Remarkably, this specimen recapitulated stereocilia stunting and extra rows observed in *Gnai3^{neo}*, *Gnai1^{neo}; Gnai3^{neo}* and *LSL-myc:ptxA* models (**Figure 2A-F** [\[4\]](#)), but apparently also an inversion of

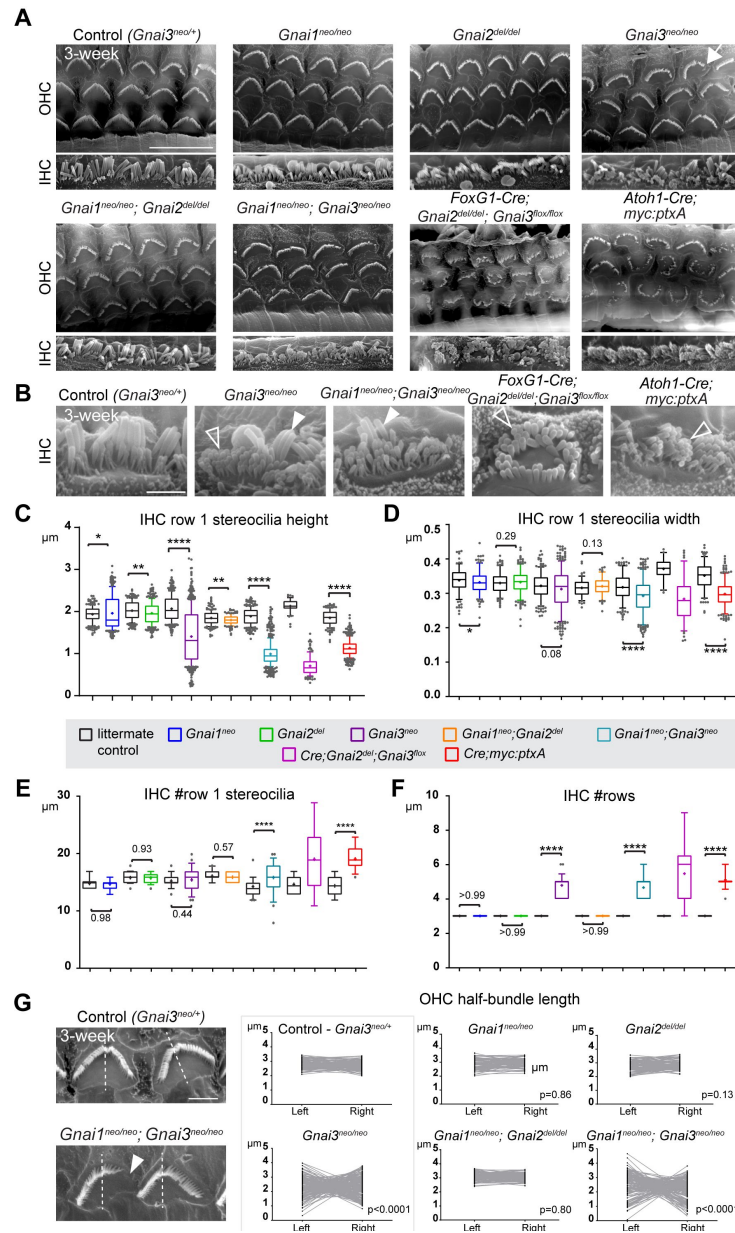


Figure 2.

Individual GNAI proteins make different contributions to hair bundle development.

A-B) SEM images of representative OHCs (A) and IHCs (B) in 3-week old animals at the cochlear first turn. *Gnai1^{neo}*, *Gnai2^{del}*, and *Gnai1^{neo}; Gnai2^{del}* mutants show apparently normal hair bundle morphology in both HC types. In contrast, *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutants show numerous defects in both HC types, including truncated hair bundles in OHCs (arrow), as well as supernumerary rows of stunted (hollow arrowheads) or longer stereocilia of variable height (full arrowheads) in IHCs. In addition, in *FoxG1-Cre; Gnai2^{del}; Gnai3^{lox}* and *Atoh1-Cre; LSL-myc:ptxA* mutants, OHC1-2s are severely misoriented. **C-F**) Quantification of various hair bundle features in 3-week old IHCs at the cochlear first turn. Each mutant strain is compared to littermate controls (in black; exact genotypes detailed in Source Data file). At least 3 animals, 17 IHCs and 108 stereocilia are represented per condition, except for *FoxG1-Cre; Gnai2^{del}; Gnai3^{lox}* where we could only obtain a single animal due to postnatal lethality. Mann-Hitney 8 test; p<0.0001, p<0.001, p<0.01, p<0.05; non-significant p-values are indicated. Detailed cohort sizes and statistics can be found in the Source Data file. **G**) SEM images of representative OHCs showing a truncated hair bundle (arrowhead). Lengths for the left and right wing of the hair bundle were measured and plotted as paired values for the same OHC. A littermate control graph is only shown for *Gnai3^{neo}* mutants (*Gnai3^{neo/+}* littermate controls). Littermate control graphs for the other mutants can be found in Fig. 2 Supp. 1G. p-values are for a F-test of variance of pooled left and right wing lengths compared to littermate controls. At least 3 animals and 88 OHCs are represented per genotype. Only *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutants show truncated hair bundles and a significant p value (p<0.05). Scale bars are 10μm (A) and 2μm (B, G).

OHC1-2 only observed to date in *LSL-myc:ptxA* and *Gpr156* mutants (**Figure 2A** [↗](#); see also below, **Figure 8** [↗](#) and **Figure 8 Supp. 1** [↗](#)) (Kindt et al, 2021 [↗](#)). IHC quantifications uncovered a $Gnai3^{neo} < Gnai1^{neo}; Gnai3^{neo} < LSL-myc:ptxA < FoxG1-Cre; Gnai2^{del}; Gnai3^{flox}$ allelic series along which a) defects increased in severity, as manifested by increased variability and decreased averages (row 1 height and width; **Figure 2C-D** [↗](#)), and b) new defects appeared (excess row 1 stereocilia in *Gnai1^{neo}*; *Gnai3^{neo}* and more affected models; **Figure 2E** [↗](#)). In general, this phenotypic series moves towards increasingly immature-looking hair bundles, and increasingly mimics severe hair bundle defects in *Gpsm2* mutants (Mauriac et al, 2017 [↗](#); Tadeney et al, 2019 [↗](#); Tarchini et al, 2016 [↗](#)).

To quantify and compare hair bundle truncations in OHCs, we measured the length of half-bundle ‘wings’ on each side of the central vertex and plotted paired left and right values for each OHC (**Figure 2G** [↗](#); **Figure 2 Supp. 1G** [↗](#)). We tested length variance using mutant strains where hair bundles retained a recognizable vertex (thus excluding *LSL-myc:ptxA* and *FoxG1-Cre; Gnai2^{del}; Gnai3^{flox}* double mutants). In *Gnai1^{neo}*, *Gnai2^{del}* and *Gnai1^{neo}; Gnai2^{del}* mutants, the two wings of each hair bundle had similar lengths and variance was similar to control littermates. In contrast, variable truncation of one wing in *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutants resulted in significantly higher length variance than controls (**Figure 2G** [↗](#); **Figure 2 Supp. 1G** [↗](#)). In both *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutants, 49% of hair bundles had wings of more different lengths than the worst OHC outlier in littermate controls (see Source Data file).

In conclusion, GNAI proteins are not equally involved in hair bundle morphogenesis, with GNAI3 playing a particularly prominent role. GNAI2 makes a clear contribution since stereocilia defects increase in severity when GNAI loss extends from GNAI3 to both GNAI2 and GNAI3. These results confirm previous conclusions (Beer-Hammer et al, 2018 [↗](#)). In addition, we largely rule out that GNAO is involved in apical HC differentiation, but uncover a possible contribution from GNAI1. Of note, as *Gnai3^{neo}* is in mixed (129S1/SvImJ; C57BL/6J) whereas *Gnai1^{neo}; Gnai3^{neo}* is in pure (129S1/SvImJ) genetic background (**Supp. Table 1** [↗](#)), we cannot rule out that the more severe defects in *Gnai1^{neo}; Gnai3^{neo}* double mutants originate from genetic modifiers in 129S1/SvImJ. Finally, the one adult specimen obtained for *FoxG1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}* suggests that GNAI2 can fully compensate for the combined loss of GNAI1 and GNAI3 in *Gnai1^{neo}; Gnai3^{neo}* double mutants at embryonic stages, and secure proper OHC1-2 orientation in the EMX2>GPR156>GNAI signaling cascade. This important point is verified and expanded below where neonate HCs are analyzed.

Only GNAI3 is required for normal auditory brainstem thresholds yet other GNAI proteins participate

To pair morphological defects with auditory function, we next tested Auditory Brainstem Response (ABR) in mutants and control littermates at 3 to 4 weeks of age. Anesthetized animals were presented with pure tone stimuli of decreasing sound pressure intensity (dB SPL) at 8, 16, 32 and 40 kHz and ABRs were recorded with a subcutaneous probe (see Methods). Mirroring their overtly normal apical HC morphology, *Gnai1*, *Gnai2* and *Gnai1; Gnai2* mutants showed thresholds comparable to littermate controls at all frequencies (**Figure 3A-C** [↗](#)). Similarly, constitutive (*Gnao1^{neo}*) or conditional (*Atoh1-Cre; Gnao1^{flox}*) inactivation of GNAO did not alter ABR thresholds (**Figure 3 Supp. 1A-B** [↗](#)). In contrast, *Gnai3^{neo}* mutants were profoundly deaf at 32 and 40kHz and displayed significantly elevated thresholds at 8 and 16 kHz compared to littermate controls (**Figure 3D** [↗](#)). *Gnai1^{neo}; Gnai3^{neo}* double mutants shared a similar ABR profile as *Gnai3^{neo}* single mutants, with apparently higher thresholds at 8 and 16 kHz although these two strains were not compared as littermates (**Figure 3D** [↗](#)). Littermate controls for *Gnai1^{neo}; Gnai3^{neo}* double mutants were homozygote for *Gnai1^{neo}* (*Gnai1^{neo/neo}*; *Gnai3^{neo/+}*) and had apparently higher thresholds than littermate controls for *Gnai3^{neo}* mutants (*Gnai3^{neo/+}*) whereas loss of GNAI1 did not impact

auditory thresholds on its own (**Figure 3A**). These results match more severe hair bundle defects when GNAI1 is inactivated along with GNAI3 (**Figure 2C-F**), and here again, could reflect either a limited role for GNAI1 in hearing or differences in genetic background.

In summary, we confirm that GNAI3, but not GNAI2, is essential for proper auditory thresholds, as previously proposed (Beer-Hammer et al, 2018). We raise the possibility that GNAI1 may have a limited role, and clarify that GNAO does not participate. Beer-Hammer and colleagues previously showed that inactivating GNAI2 worsens hearing loss in the *Gnai3* null background, as tested in *FoxG1-Cre; Gnai2^{flox/flox}; Gnai3^{flox/flox}* adults (Beer-Hammer et al, 2018). Using a comparable yet distinct model (*FoxG1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}*), we were largely unable to obtain young adults and thus could not perform ABR. This suggests that our model is more severely affected, and probably lacks ABRs similar to the *FoxG1-Cre; Gnai2^{flox/flox}; Gnai3^{flox/flox}* (Beer-Hammer et al, 2018) and *LSL-myc:ptxA* (Tarchini et al, 2016) models.

Distribution of individual GNAI proteins in neonate hair cells

Next, we attempted to detect individual GNAI/O proteins in neonate HCs and define how they localize. Because GNAI/O proteins are close paralogs, validating and using specific antibodies is challenging. Instead, we used one commercial antibody raised against GNAI3 (scbt"GNAI3"; expected to detect GNAI3>GNAI1>GNAI2) and one antibody raised against GNAI2 (pt"GNAI2"; expected to detect GNAI2>GNAI1>GNAI3; see Methods) and systematically immunolabeled our collection of mouse models to tease apart protein-specific behavior.

Both antibodies produced the familiar GNAI pattern of enrichment at the bare zone and at row 1 stereocilia tips (**Figure 4**) (Tarchini et al, 2013; Tarchini et al, 2016). Both revealed generally normal GNAI enrichment in *Gnai1^{neo}* (**Figure 4A**), *Gnai2^{del}* (**Figure 4B**), and *Gnai1^{neo}; Gnai2^{del}* double mutants (**Figure 4C**). This indicates that pt"GNAI2" is not specific for GNAI2, can also detect GNAI3, and should thus be able to detect GNAI1, a better target than GNAI3. In *Gnai3^{neo}* single and *Gnai1^{neo}; Gnai3^{neo}* double mutants, both antibodies still revealed GNAI protein at the bare zone and at stereocilia tips although some HCs displayed incomplete enrichment (**Figure 4D-E**; further detailed below). This indicates that scbt"GNAI3" is not specific for GNAI3, can also detect GNAI2, and should thus be able to detect GNAI1, a better target than GNAI2. Finally, neither antibody detected consistent signal over background in *Gnai2; Gnai3* double mutants (**Figure 4F**; *FoxG1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}*). Together, these results indicate that a) GNAI2 and GNAI3 share the same polarized distribution pattern at the bare zone and at stereocilia tips, and b) GNAI1 is either present in very low amounts hidden by background signal, or absent at the HC apical surface. Loss of GNAO in the *Gnao1^{neo}* or *Atoh1-Cre; Gnao1^{flox}* models did not alter signals obtained with scbt"GNAI3" (**Figure 4 Supp. 1A-B**). Moreover, a GNAO antibody produced unpolarized apical signals that we deemed unspecific because they were unchanged in *Atoh1-Cre; Gnao1^{flox/flox}* mutants (**Figure 4 Supp. 1C**). GNAO thus likely does not contribute to HC polarization, as also suggested by normal hair bundles and normal ABR thresholds in *Gnao1* mutants.

GNAI2 only partially spans the bare zone and stereocilia tips and partially rescues hair bundle development in postnatal hair cells lacking GNAI3

We next took a closer look at incomplete GNAI enrichment in *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutant HCs (**Figure 4D-E**). In both models, we observed an identical outcome at the P0 cochlear base where remaining GNAI was unable to fully and consistently occupy the HC sub-domains where GNAI3 was missing (**Figure 5A-B**; bare zone, arrows; stereocilia tips, arrowheads). In *Gnai1^{neo}; Gnai3^{neo}* double mutants (**Figure 5B**), the GNAI protein detected is by default GNAI2 and, based on results above (**Figure 4**), we conclude that GNAI2 likely also forms the bulk of

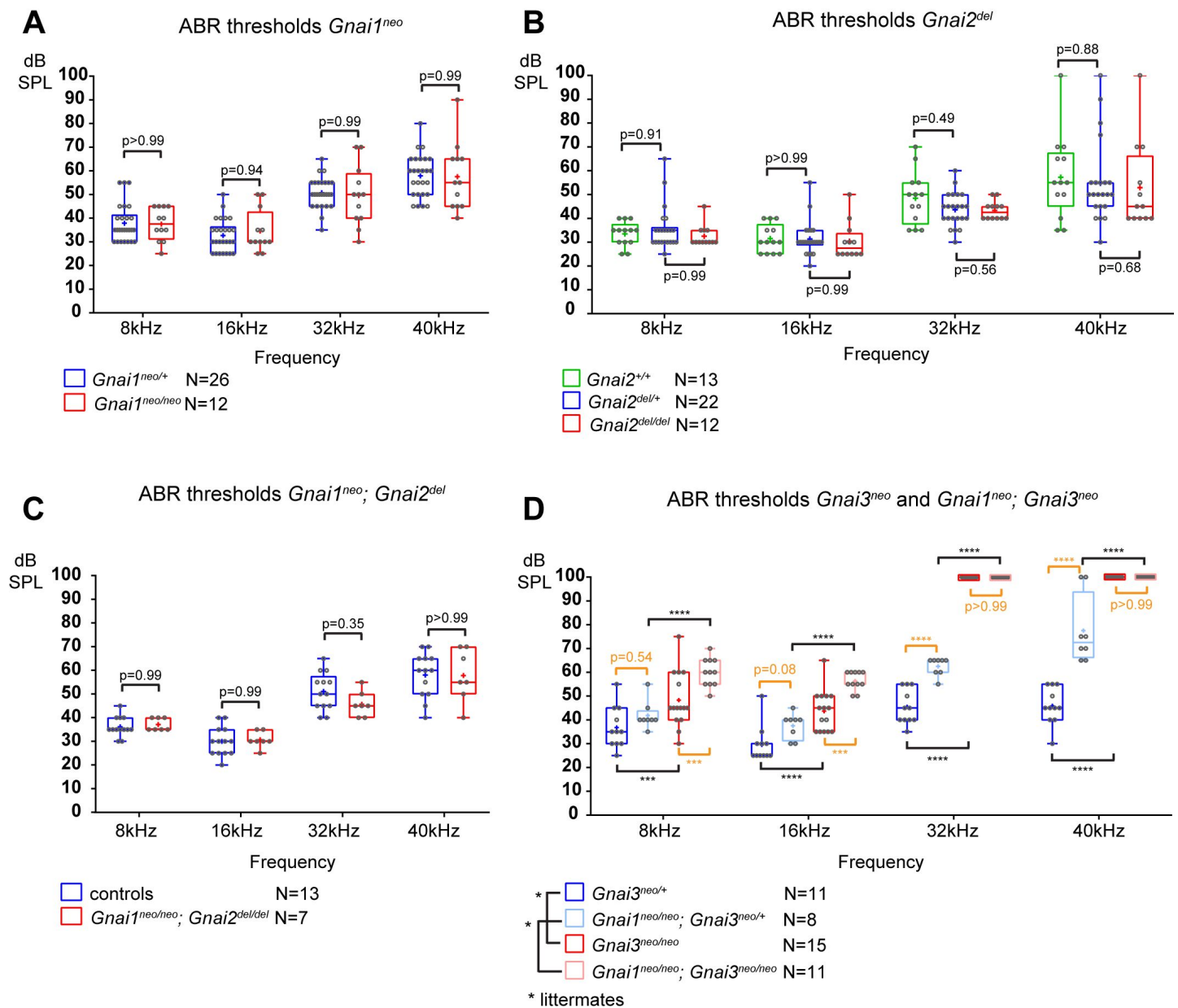


Figure 3.

Loss of GNAI3 leads to profound deafness at high frequencies.

A-D) ABR thresholds at 8, 16, 32, and 40 kHz for *Gnai1^{neo}* (A), *Gnai2^{del}* (B), *Gnai1^{neo}; Gnai2^{del}* (C), and *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* (D) mutants tested between P21 and P29. Boxplots are framed with 25-75% whisker boxes where exterior lines show the minimum and maximum values, the middle line represents the median, and + represent the mean. A plotted value of 100 dB indicates that animals did not respond to 90 dB stimuli. In (C), controls are a pool of *Gnai1^{+/+}; Gnai2^{del/+}*, *Gnai1^{neo/+}; Gnai2^{+/+}* and *Gnai1^{neo/+}; Gnai2^{del/+}* animals. N indicates the number of animals tested. Two-way ANOVA with Sidak's multiple comparison. $p < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *; non-significant p-values are indicated. P-values in orange were obtained comparing non-littermate animals and suggest possibly raised thresholds when GNAI1 is inactivated in addition to GNAI3 (see text). kHz, kilohertz, dB SPL, decibel sound pressure level.

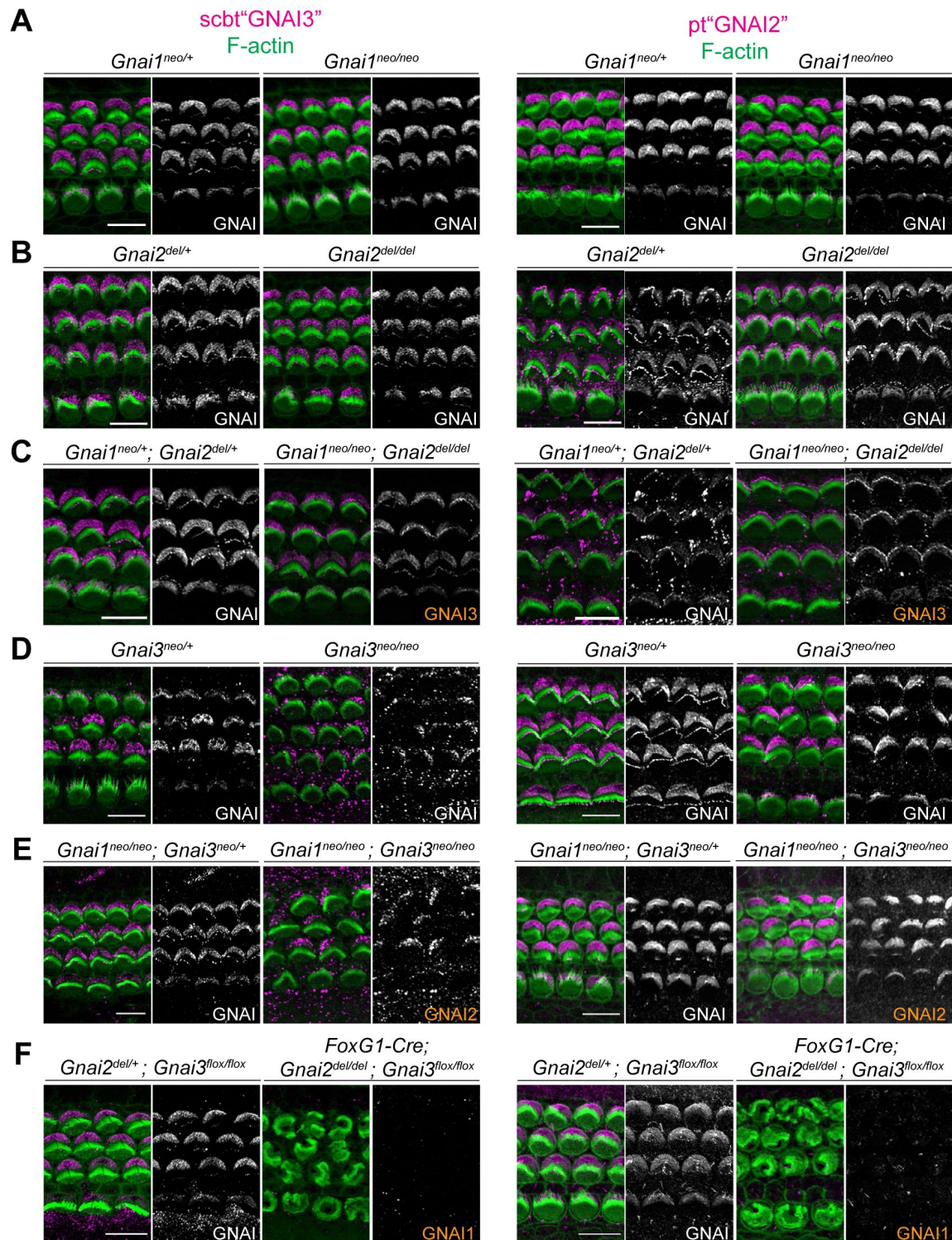


Figure 4.

Systematic immunolabeling of GNAI proteins in *Gnai* mutant strains.

A-F) Two different antibodies (*scbt*⁺"GNAI3" and *pt*⁺"GNAI2") were used to label the auditory epithelium at P0-P3. Neither antibody is specific for its protein target, as *scbt*⁺"GNAI3" is able to detect GNAI2 (E) and *pt*⁺"GNAI2" is able to detect GNAI3 (C). Note how no apical GNAI signal is visible with either antibody in *Gnai2*; *Gnai3* double mutants (F). When the identity of the GNAI protein detected is unambiguous based on the sample genotype, it is made explicit in orange. Scale bars are 10µm.

remaining GNAI in *Gnai3^{neo}* mutants (**Figure 5A**). Because in all cases GPSM2 co-localized with GNAI2 (**Figure 5A-B**), these results demonstrate that GNAI2, and not only GNAI3, can form a complex with GPSM2 in HCs. Intriguingly, the absence of GPSM2-GNAI2 on one side of the bare zone at P0 appeared to coincide with its absence at stereocilia tips on the corresponding side (**Figure 5A-B**). We thus quantified GNAI2 intensity at the bare zone and at stereocilia tips in half-OHCs in *Gnai1^{neo}; Gnai3^{neo}* mutants. While as expected control OHCs showed little variation in GNAI enrichment in either compartment (**Figure 5 Supp. 1A**), mutant OHCs showed highly variable signals that were significantly correlated between the bare zone and tips in the same half-OHC (**Figure 5C**). Analyzing HCs at different stages and tonotopic positions (gradient of increasing HC maturity from cochlear apex to base) clarified that GNAI2 is progressively unable to compensate for missing GNAI3. At E18.5, the GPSM2-GNAI2 complex could still occupy the totality of the bare zone in *Gnai1^{neo}; Gnai3^{neo}* mutants (**Figure 5 Supp. 1B**), suggesting that GNAI2 fully compensates for the loss of GNAI3 in embryonic HCs. Partial complementation by GNAI2 observed at P0 (**Figure 5A-C**) evolved into a lack of complementation at P6 at the cochlear base, where GNAI2 was no longer detected at the tips of stunted stereocilia in mutant IHCs (**Figure 5D**). In contrast, more immature P6 IHCs at the cochlear mid position retained partial GNAI2 enrichment correlated between the bare zone and tips (**Figure 5E**), as seen at P0 (**Figure 5A-B**). Time and position-based differences suggest that GNAI2 is progressively unable to occupy the full bare zone and tip compartments during HC differentiation.

The progressive inability of GNAI2 to cover for GNAI3 in individual HCs helps explain the unique profile of apical surface defects in *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutant HCs. Loss of GPSM2-GNAI2 in one wing of the OHC hair bundle led to stereocilia degeneration by P8 (**Figure 5F**). One-sided loss of global GNAI function at stereocilia tips is thus likely the origin of truncated hair bundle wings observed in adults *Gnai3^{neo}* and *Gnai1^{neo}; Gnai3^{neo}* mutant OHCs (**Figure 2G**). We divided the P8 OHC apical surface in two halves based on the position of the basal body at the hair bundle vertex, and measured the length of each hair bundle wing as well as the total apical surface area in the same half in *Gnai1^{neo}; Gnai3^{neo}* mutants (**Figure 5F**). We found a significant correlation between these two values (**Figure 5G**; control graph in **Figure 5 Supp. 1C**), providing new evidence that loss of stereocilia prompts a corresponding loss of flat HC surface area on the same OHC side (Etournay et al, 2010). Finally, we asked whether in time GNAI2 is lost at all stereocilia tips and along the entire cochlea in *Gnai1^{neo}; Gnai3^{neo}* mutants. This proved not to be the case, as GNAI2 could still be detected at stereocilia tips in P28 OHCs and IHCs, although in low and variable amounts compared to GNAI tip signals in littermate controls (**Figure 5 Supp. 1D-E**).

In conclusion, GNAI2 still provides a low dose of GNAI protein at the tip of stereocilia that do not degenerate when GNAI3 is missing. Variable GNAI2 amounts likely explain why IHCs stereocilia have variably reduced heights in absence of GNAI3 (**Figure 2B-C**; **Figure 5D-E**), unlike in *Gpsm2* or *LSL-myc:ptxA* mutants where they are more uniformly stunted (Beer-Hammer et al, 2018; Mauriac et al, 2017; Tadenev et al, 2019; Tarchini et al, 2016). Interestingly, correlated loss of GNAI2 signal at the bare zone and at stereocilia tips on the same HC side adds to previous evidence suggesting that bare zone enrichment is essential for GPSM2-GNAI transfer to adjacent row 1 stereocilia (Akturk et al, 2022; Jarysta & Tarchini, 2021; Tarchini et al, 2016).

Combined loss of GNAI2 and GNAI3 delays and de-polarizes bare zone expansion with drastic consequences on stereocilia distribution

Since GNAI2 and GNAI3 show functional redundancy and are the most important GNAI/O proteins for hair bundle differentiation, we next focused on characterizing early HC development in *Foxg1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}* mutants and comparing defects to those observed previously with Pertussis toxin. Unlike at adult stage, *Gnai2; Gnai3* double mutants were obtained in close to Mendelian proportion at P0. For this purpose, we used the new *DIO-ptxA* allele in case the myc tag

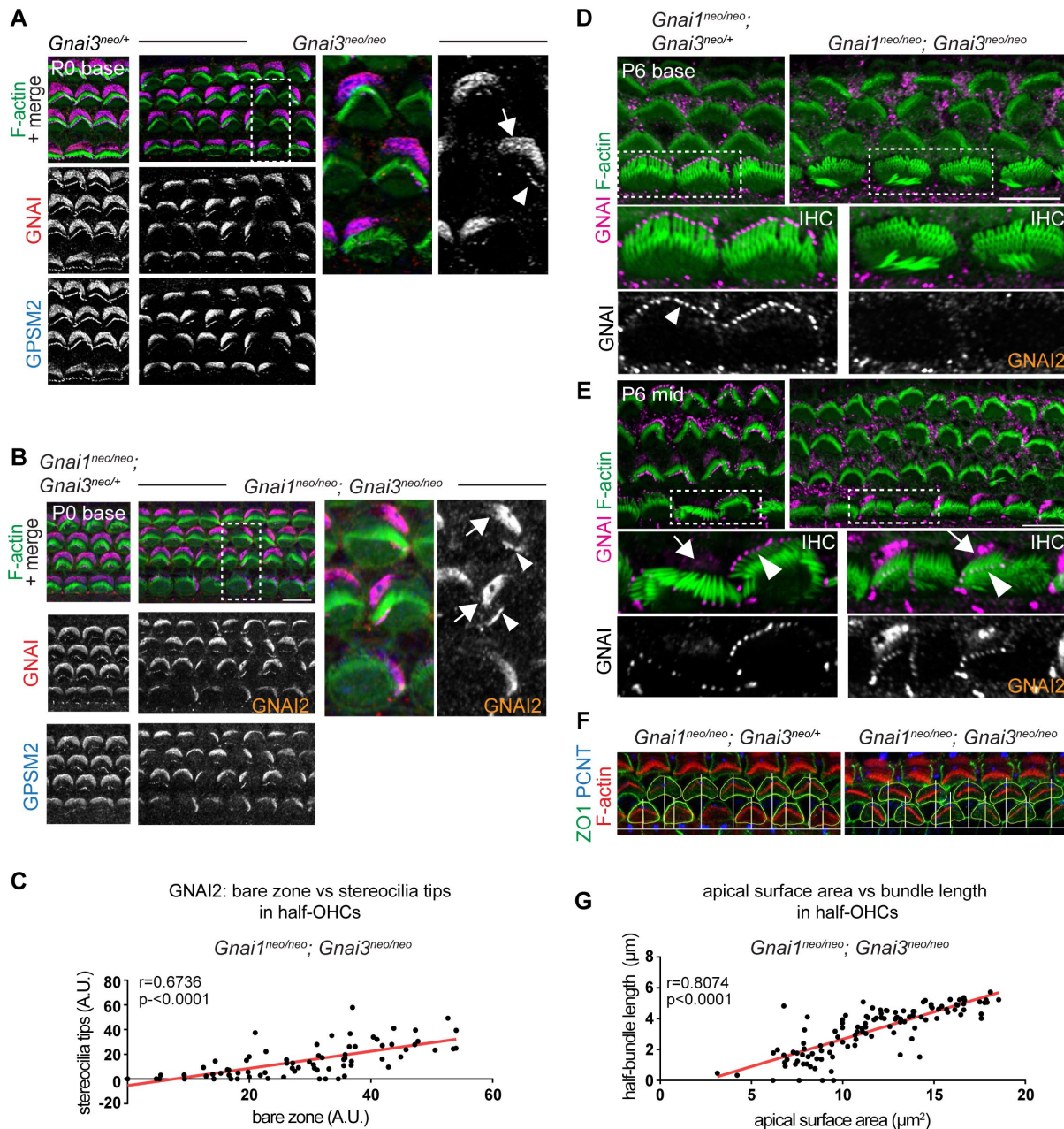


Figure 5.

GNAI2 only partially rescues the loss of GNAI3 in individual postnatal hair cells.

A-B) GNAI (pt“GNAI2” antibody; see Fig. 4) and GPSM2 co-immunolabeling in P0 *Gnai3^{neo/+}* (A) and *Gnai1^{neo/neo}; Gnai3^{neo/+}* (B) animals at the cochlear base. Boxed regions are magnified on the right. In both mutants, incomplete GNAI patterns are observed at the bare zone (arrow) and stereocilia tips (arrowheads). Remaining GNAI signals are specifically GNAI2 in (B). **C)** Correlation plot of GNAI signal intensity at the bare zone and tips in half-OHCs at the P2 cochlear first turn. Presence or loss of GNAI is remarkably correlated spatially between bare zone and tips in the same half-HC. N=3 animals, n=37 OHCs, Pearson correlation with best fit (red line; plot for control littermates can be found in Fig. 5 Supp. 1A). **D-E)** GNAI (pt“GNAI2” antibody) immunolabeling in P6 *Gnai1^{neo/neo}; Gnai3^{neo/+}* animals. Boxed IHC regions are magnified below. Loss of GNAI2 progresses with HC differentiation, with largely lost IHC signals at the P6 cochlear base (D) but partial rescue on one side of the cell at the P6 first turn (E), as observed at the cochlear base at P0 (A-B). **F-G)** ZO1 (apical junctions) and pericentrin (PCNT; basal body) immunolabeling in P8 OHCs. The position of the basal body was used to determine the vertex (middle) of the original hair bundle and to draw a radial line separating each OHC into two halves (F). The length of each hair bundle wing (y axis) is graphed in relation to the corresponding apical surface area (x axis) in the same half-OHC (G). Truncated OHC wings correlate with reduced apical membrane area on the same side. N=3 animals, n=58 OHCs, Pearson correlation with best fit (red line; plot for control littermates can be found in Fig. 5 Supp. 1C). A.U., arbitrary unit. Scale bars are 10µm.

hindered ptxA activity in the *LSL-myc:ptxA* allele. We started with validating the new *Gnai3^{flox}* and *DIO-ptxA* alleles (**Figure 2 Supp. 1B-C**). As expected, GNAI signals at the bare zone and stereocilia tips were normal in *Gnai3^{flox/flox}* homozygotes but showed a distinctive incomplete pattern along with stunted stereocilia upon Cre recombination (**Figure 6 Supp. 1A**), as in constitutive mutants (**Figure 5A**). The new *DIO-ptxA* model produced in fact identical apical HC defects to the earlier *LSL-myc:ptxA* strain in single HCs (Kindt et al, 2021; Tarchini et al, 2016) (**Figure 6 Supp. 1B**). The fraction of inverted OHC1 in the *DIO-ptxA* model was lower than in the *LSL-myc:ptxA* model when using the later *Atoh1-Cre* (**Figure 6 Supp. 1C-D**), but identically encompassed 100% of OHC1 with *FoxG1-Cre*. Similar apical HC defects between strains indicate that the N-terminal myc and mild *R26* promoter do not limit ptxA activity in *LSL-myc:ptxA*. For comparison purposes, we used the *FoxG1-Cre* driver to inactivate GNAI2/GNAI3 and to express ptxA, the same driver used by Beer-Hammer and colleagues (Beer-Hammer et al, 2018).

Inactivating GNAI2 and GNAI3 abolished the bare zone at E17.5 based on the absence of GNAI labeling that was obvious in controls, and abnormally uniform F-actin signals at the HC surface (**Figure 6A-B**; arrows point to the bare zone). In contrast, E17.5 *DIO-ptxA* HCs had developed a distinct bare zone (**Figure 6C**, arrows). Measuring its surface area by HC type confirmed that the bare zone is virtually absent in conditional *Gnai2*; *Gnai3* double mutants and reduced in ptxA-expressing OHCs but not IHCs (**Figure 6D**). By P0, most *Gnai2*; *Gnai3* mutant HCs at the cochlear base had developed a region lacking microvilli or stereocilia, but its position at the apical surface was highly irregular, complementing extremely dysmorphic hair bundles (**Figure 6E-F**; arrows point to bare regions). In contrast, P0 *DIO-ptxA* HCs displayed largely coherent hair bundles and a distinct bare zone (**Figure 6G**, arrows). Quantifications revealed a significantly reduced bare area in P0 *Gnai2*; *Gnai3* mutants compared to littermate controls (**Figure 6H**), showing that bare zone emergence and expansion is greatly delayed and deregulated in this model (compare P0 in **Figure 6H** and E17.5 in **Figure 6D**). In line with delayed bare zone expansion upon inhibiting GNAI function, bare zone surface area was back to normal in P0 *DIO-ptxA* HCs, except in the least mature OHC3 (compare P0 in **Figure 6H** and E17.5 in **Figure 6D**).

These results best illustrate to date the importance of GPSM2-GNAI for bare zone emergence, expansion and polarized positioning. While both mutant models consistently delay bare zone expansion, differences in timing and severity suggest that ptxA expression does not achieve as extended a loss of GNAI function as the *Gnai2*; *Gnai3* double mutant. This is further underscored by severe stereocilia distribution defects observed in *Gnai2*; *Gnai3* but not *DIO-ptxA* mutants. As reported previously (Kindt et al, 2021; Tarchini et al, 2013; Tarchini et al, 2016), the orientation of OHC1-2 expressing ptxA was reversed compared to controls (**Figure 6C**, G), and we expected to observe a similar pattern of misorientation in *Gnai2*; *Gnai3* mutants. However, HC orientation proved challenging to assess in *Gnai2*; *Gnai3* mutants due to highly dysmorphic hair bundles (**Figure 6A-B**; E-F). We used acetylated tubulin (AcTub) and pericentrin (PCNT) as markers for the kinocilium and the position of the basal body, respectively. This showed that in *Gnai2*; *Gnai3*, but not in *DIO-ptxA* mutants, the basal body and kinocilium were sometimes in an approximately central position surrounded partially or entirely by stereocilia (**Figure 6E-F**). Rounded perinatal hair bundles are a hallmark of HCs where the early off-center migration of the basal body, hence symmetry breaking, is defective.

To distinguish defects affecting symmetry-breaking versus HC orientation and to uniformize analysis of HC polarization, we next used the position of the basal body at the base of the kinocilium to derive both HC eccentricity and HC orientation. This strategy helped compare the two mouse models, and identified two early functions for GNAI before its association with GPSM2 for stereocilia elongation.

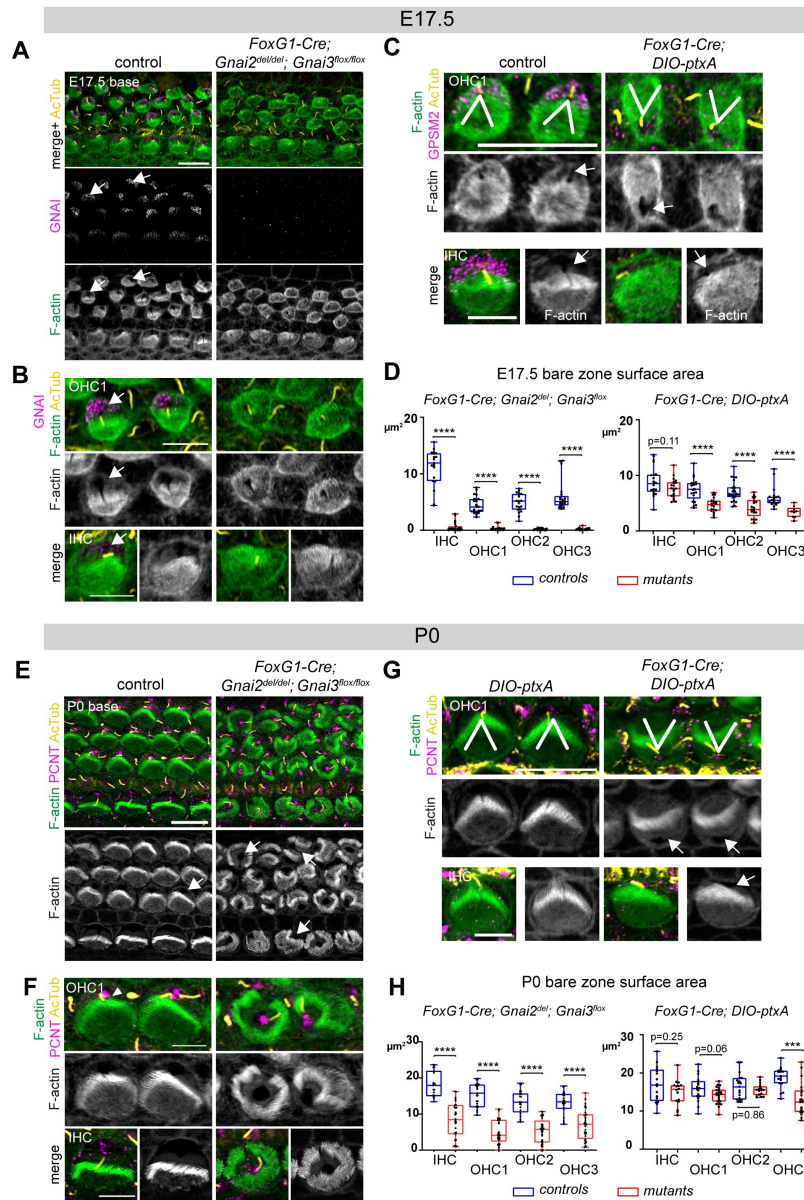


Figure 6.

Delayed bare zone expansion and severely dysmorphic hair bundles in absence of GNAI2 and GNAI3.

A-B) GNAI (scbt“GNAI3” antibody, see Fig. 4) and acetylated tubulin (AcTub) co-immunolabeling at the E17.5 cochlear base. Note how F-actin labeling (phalloidin) reveals a polarized bare zone marked by GNAI (arrows) in control but not in *Gnai2*; *Gnai3* double mutants. **C)** GPSM2 and AcTub co-immunolabeling at the E17.5 cochlear base. In contrast to *Gnai2*; *Gnai3* double mutants, *FoxG1-Cre; DIO-ptxA* mutants have a polarized bare zone (arrows) despite OHC1-2 adopting a reversed orientation (V brackets indicate orientation). GPSM2 marks the bare zone in controls and is reduced in mutants. **D)** Graphs of bare zone surface area in E17.5 HCs at the cochlear base. *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/flox}*: controls (*Gnai2^{del/+}; Gnai3^{flox/+}* and *Gnai2^{del/+}; Gnai3^{flox/flox}*) N=3 animals, n=19 IHC, 23 OHC1, 19 OHC2, 21 OHC3; mutants N=3, n=23 IHC, 24 OHC1, 23 OHC2, 24 OHC3. *FoxG1-Cre; DIO-ptxA*: controls (*DIO-ptxA*) N=3, n=18 IHC, 18 OHC1, 21 OHC2, 18 OHC3; mutants N=3, n=21 IHC, 20 OHC1, 21 OHC2, 9 OHC3. **E-G)** Pericentrin (PCNT) and AcTub co-immunolabeling at P0 at the cochlear base. Unlike at E17.5 (A-B, D), most P0 *Gnai2*; *Gnai3* double mutant HCs have a bare region (E-F, arrows). This bare region is unpolarized and its abnormal shape reflects aberrant stereocilia distribution. In sharp contrast, *ptxA* mutants have normally-shaped hair bundles and bare zones despite OHC1-2 adopting a reversed orientation (G). **H)** Graphs of bare zone surface area in P0 HCs at the cochlear base. *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/+}*: controls (*Gnai2^{del/+}; Gnai3^{flox/+}* and *Gnai2^{del/+}; Gnai3^{flox/flox}*) N=3, n=19 IHC, 23 OHC1, 21 OHC2, 21 OHC3; mutant N=3, n=21 IHC, 22 OHC1, 24 OHC2, 24 OHC3. *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/+}* N=3, n=19 IHC, 23 OHC1, 21 OHC2, 21 OHC3; mutants N=3, n=16 IHC, 24 OHC1, 18 OHC2, 19 OHC3. **D, H:** Mann-Whitney U test; p<0.0001 ****, p<0.001 ***, p<0.01 **, p<0.05 *; non-significant p-values are indicated. All *ptxA* samples are heterozygotes (*R26^{DIO-ptxA/+}*). Scale bars are 10μm (A, C (OHC), E, G (OHC)) and 5μm (B, C (IHC), F, G (IHC)).

GNAI proteins drive the off-center migration of the basal body

We used the position of the basal body to measure HC eccentricity as a metric for cytoskeleton asymmetry. Eccentricity was calculated as a ratio reporting how far away from the cell center the basal body was positioned (see schematic in [Figure 7](#)). A perfectly symmetrical HC with a central basal body would thus have an eccentricity ratio close to 0 whereas a ratio close to 1 would indicate that the off-center basal body is juxtaposed to the apical junction. Because HC maturation progresses in time and along the tonotopic axis (cochlear apex to base gradient of increasing maturity), we measured eccentricity at E17.5 (base and mid cochlear position) and at P0 (base, mid and apex) to understand how eccentricity progressed at the HC population level.

Control HCs at E17.5 had already broken symmetry at the mid position, with eccentricity averaging ~0.5 and increasing at the more mature base position ([Figure 7A](#)). At E17.5 mid and base positions, *Gnai2*; *Gnai3* mutants showed significantly reduced eccentricity, suggesting a delay in the off-center migration of the basal body. We arbitrarily defined 0.25 as a threshold below which HCs were considered “symmetrical”. Symmetrical HCs were not observed in controls but represented up to ~29% of IHCs and ~34% of OHCs in mutants at E17.5 (detailed in [Figure 7A](#) by stage, position and HC type, red highlights). By P0, the proportion of symmetrical cells in mutants had decreased at all positions for OHCs (3-15%), but remained high for IHCs (21.5%; [Figure 7A](#)). P0 OHC eccentricity generally remained lower in mutants than in controls, with a trend towards less significant differences in OHCs at the cochlear base where HCs are most mature. In contrast, IHCs did not appear to recover by P0, as eccentricity was much lower than in controls, even at the cochlear base.

The situation was radically different in the *DIO-ptxA* model. First, no symmetrical HC was observed at any stage or position in mutants ([Figure 7B](#)). In fact, eccentricity was instead higher in *DIO-ptxA* OHCs compared to littermate controls, with a tendency for this difference to become less significant along with OHC differentiation (see below for interpretation). IHCs had overall a normal distribution of eccentricity compared to controls in the *DIO-ptxA* model.

Defects in bare zone expansion ([Figure 6D](#), H) can help explain the distinct eccentricity defects in the *DIO-ptxA* and *Gnai2*; *Gnai3* mouse models. At E17.5 and P0, the position of the basal body is the sum of two opposite movements ([Figure 1A](#)): a) the early off-center migration that brings it in the vicinity of the lateral junction, and b) the subsequent relocation towards the cell center that brings it in contact with the forming hair bundle upon bare zone expansion ([Tarchini et al., 2013](#)). In *DIO-ptxA*, we interpret transiently increased eccentricity in OHCs ([Figure 7B](#)) as the outcome of normal off-center basal body migration yet delayed bare zone expansion ([Figure 6D](#), H). In *Gnai2*; *Gnai3* mutants, however, decreased eccentricity in OHCs ([Figure 7A](#)) stems from defective basal body off-center migration compounded with a first absent (E17.5), and then reduced (P0), bare region ([Figure 6D](#), H). When an unpolarized bare region eventually emerges in *Gnai2*; *Gnai3* mutants ([Figure 6H](#)), it impacts basal body position without directionality, leading to highly variable eccentricity values compared to controls ([Figure 7A](#)), and thus variably dysmorphic hair bundles. The apparent increase in symmetrical IHCs over time in *Gnai2*; *Gnai3* mutants ([Figure 7A](#)) may result from the delayed expansion of the bare region, which will relocalize the basal body centrally in a proportion of IHCs.

In conclusion, the loss of GNAI activity achieved in *Gnai2*; *Gnai3* mutants impairs symmetry-breaking ([Figure 7](#)) before it impairs bare zone emergence and expansion ([Figure 6](#)). PtxA-based downregulation of GNAI activity is sufficient to delay bare zone expansion, but not to affect symmetry-breaking. As only ~3-15% of OHCs and ~21% of IHCs in *Gnai2*; *Gnai3* mutants are symmetrical at the P0 cochlear base, there is likely sufficient GNAI activity remaining in this model as well for most HCs to break planar symmetry.

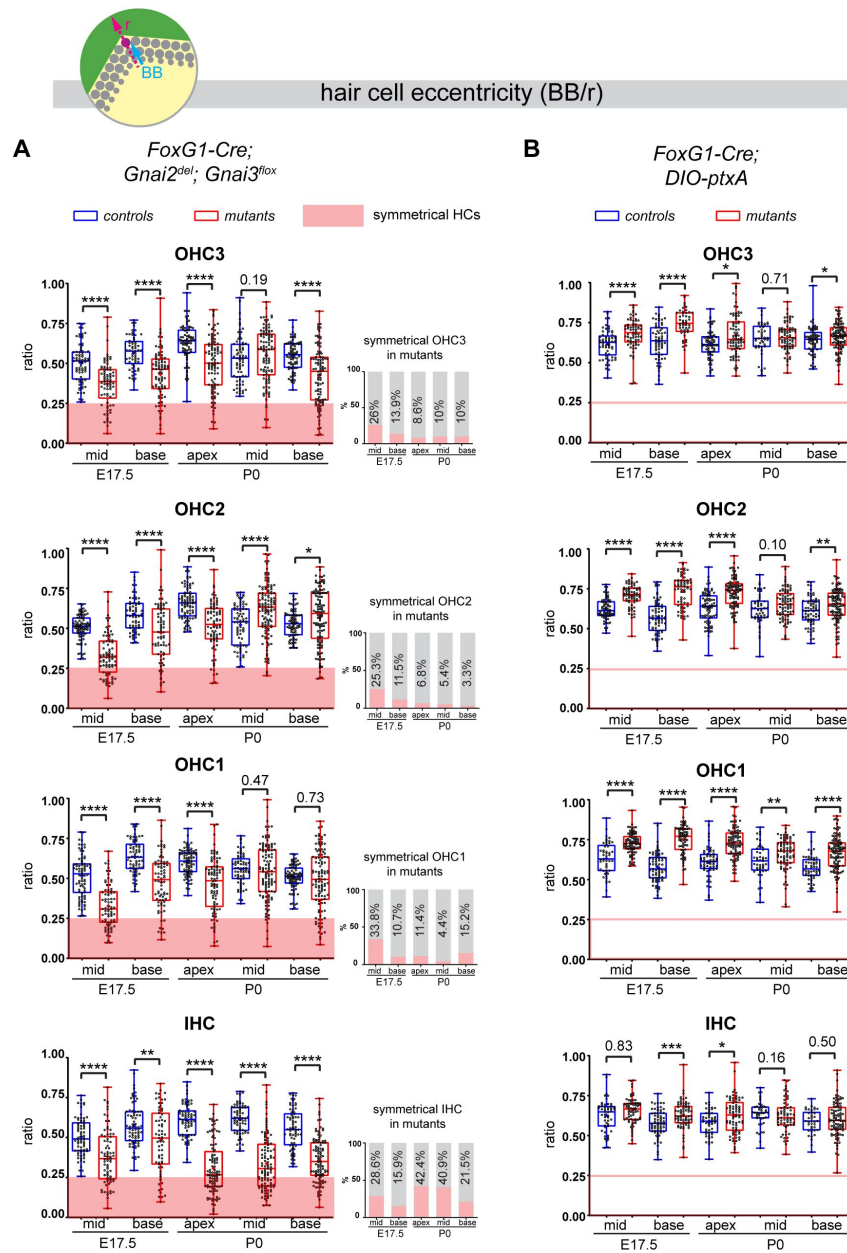


Figure 7.

Loss of GNAI2 and GNAI3 provokes hair cell eccentricity defects absent in *ptxA* mutants.

A-B) Graphs of HC eccentricity representing the position of the basal body as a ratio of the radius (BB/r, top diagram). Data cover E17.5 mid and base and P0 apex, mid and base cochlear positions for each HC type. HCs were considered symmetrical when their eccentricity ratio was lower than 0.25 (red zone). Only *FoxG1-Cre; Gnai2^{del}; Gnai3^{flox}* mutants harbor symmetrical HCs. Their proportion is indicated in the bar graphs on the right (A). Overall, the proportion of symmetrical cells tends to decrease in maturing OHCs but remains higher in IHCs. At least 3 animals and 39 cells per HC type are represented for each stage, cochlear position and genotype (detailed in the Source Data file). Controls for *FoxG1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}* are *Gnai2^{del/+}; Gnai3^{flox/+}*, *Gnai2^{del/+}; Gnai3^{flox/flox}*, *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/+}* and *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/flox}*. Controls for *FoxG1-Cre; DIO-ptxA* are *DIO-ptxA* heterozygotes. Mann-Whitney U test: $p < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *; non-significant p-values are indicated. Detailed cohort sizes and statistics can be found in Source Data file. OHCs but remains higher in IHCs. At least 3 animals and 39 cells per HC type are represented for each stage, cochlear position and genotype (detailed in the Source Data file). Controls for *FoxG1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}* are *Gnai2^{del/+}; Gnai3^{flox/+}*, *Gnai2^{del/+}; Gnai3^{flox/flox}*, *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/+}* and *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/flox}*. Controls for *FoxG1-Cre; DIO-ptxA* are *DIO-ptxA* heterozygotes. Mann-Whitney U test: $p < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$ *; non-significant p-values are indicated. Detailed cohort sizes and statistics can be found in Source Data file.

GNAI proteins orient hair cells laterally

GNAI proteins were proposed to signal downstream of the GPR156 receptor to regulate HC orientation in the auditory and vestibular organs (Jiang et al, 2017 [↗](#); Kindt et al, 2021 [↗](#)). In HCs expressing the transcription factor EMX2, GPR156 reverses the orientation of the off-center basal body migration, and thus HC orientation, compared to *Emx2*-negative HCs (Tona & Wu, 2020 [↗](#)). To date, however, the HC misorientation profile observed in *Emx2* or *Gpr156* mutants was only recapitulated using *ptxA* (Figure 1B [↗](#)). If *ptxA*-based orientation defects are physiologically relevant to GNAI function, they should be observed in *Gnai2*; *Gnai3* mutants that achieve a further loss of GNAI activity.

To test this assumption, we first excluded symmetrical HCs (eccentricity < 0.25) in the E17.5 and P0 datasets since an asymmetric cytoskeleton is pre-requisite for HCs to exhibit a defined orientation. Next, we used the position of the off-center basal body in the remaining HCs to infer their orientation. We measured the angle formed by a vector running from the HC center to the basal body relative to the cochlear longitudinal axis (α , see schematic in Figure 8 [↗](#)). Angles were plotted in circular histograms where 0° pointed to the cochlear base along the longitudinal axis, and 90° to the lateral edge.

At E17.5 at the cochlear mid position, *DIO-ptxA* already showed the graded pattern of OHC misorientation described previously, with inverted OHC1-2 and imprecisely oriented IHCs and OHC3 (Figure 8A [↗](#)). At this stage and position, *Gnai2*; *Gnai3* mutant HCs above threshold showed abnormal and highly variable orientations without a clear trend towards OHC1-2 inversion yet. Strikingly however, more mature E17.5 OHC1 at the base were largely inverted (Figure 8B [↗](#)), as also observed at P0 at all positions (Figure 8C-D [↗](#); Figure 8 [↗](#) Supp. 1A [↗](#)). OHC2 were still largely oriented laterally at the E17.5 mid (Figure 8B [↗](#)) and P0 apex and mid (Figure 8C [↗](#); Figure 8 [↗](#) Supp. 1A [↗](#)), but clearly adopted an inverted medial orientation at the P0 base (Figure 8D [↗](#)). These results thus confirm for the first time that endogenous GNAI proteins are integral to proper HC orientation. They also clarify that the level of GNAI activity required for proper HC orientation is higher than for symmetry-breaking since both *DIO-ptxA* and *Gnai2*; *Gnai3* mutants showed HC misorientation. It should be noted that concomitant deficits in HC eccentricity and the arbitrary eccentricity cut-off at 0.25 were expected to blur the misorientation pattern, explaining why OHC1-2 inversion only becomes apparent at later stages of differentiation in *Gnai2*; *Gnai3* compared to *DIO-ptxA* mutants. Interestingly, unlike in *DIO-ptxA* mutants, IHC and OHC3 were also severely misoriented in *Gnai2*; *Gnai3* mutants at all stages and positions analyzed (Figure 8 [↗](#); Figure 8 [↗](#) Supp. 1A [↗](#)). We thus conclude that a lower level of GNAI activity is sufficient to properly orient IHC and OHC3 compared to OHC1-2 (see Model Figure 9 [↗](#)).

Discussion

By examining a large collection of single and combined mutations in *Gnai* genes (*Gnai1*, *Gnai2*, *Gnai3*, *Gnao1*), we assign here three distinct and successive roles for GNAI proteins during the apical polarization of a developing HC (Figure 9 [↗](#)): a) drive the centrifugal migration of the basal body when a prospective HC breaks planar symmetry, b) orient this migration along the PCP axis in a binary manner, and c) position and elongate stereocilia during hair bundle morphogenesis. Key results in the study include demonstrating that endogenous GNAI proteins indeed serve early functions (a) and (b), since to date only hair bundle defects (c) were reported in knock-out mouse models where *Gnai* genes were directly targeted (Beer-Hammer et al, 2018 [↗](#); Mauriac et al, 2017 [↗](#)). Importantly, previous studies indicated that GNAI proteins partner with different regulators in these different functions, organizing and elongating stereocilia by binding to the scaffold GPSM2 (c), and reversing HC orientation in *Emx2*-expressing HCs via heterotrimeric signaling downstream of the GPR156 receptor (b). A GNAI regulator or mechanism for basal body off-center migration (a) remains to be identified.

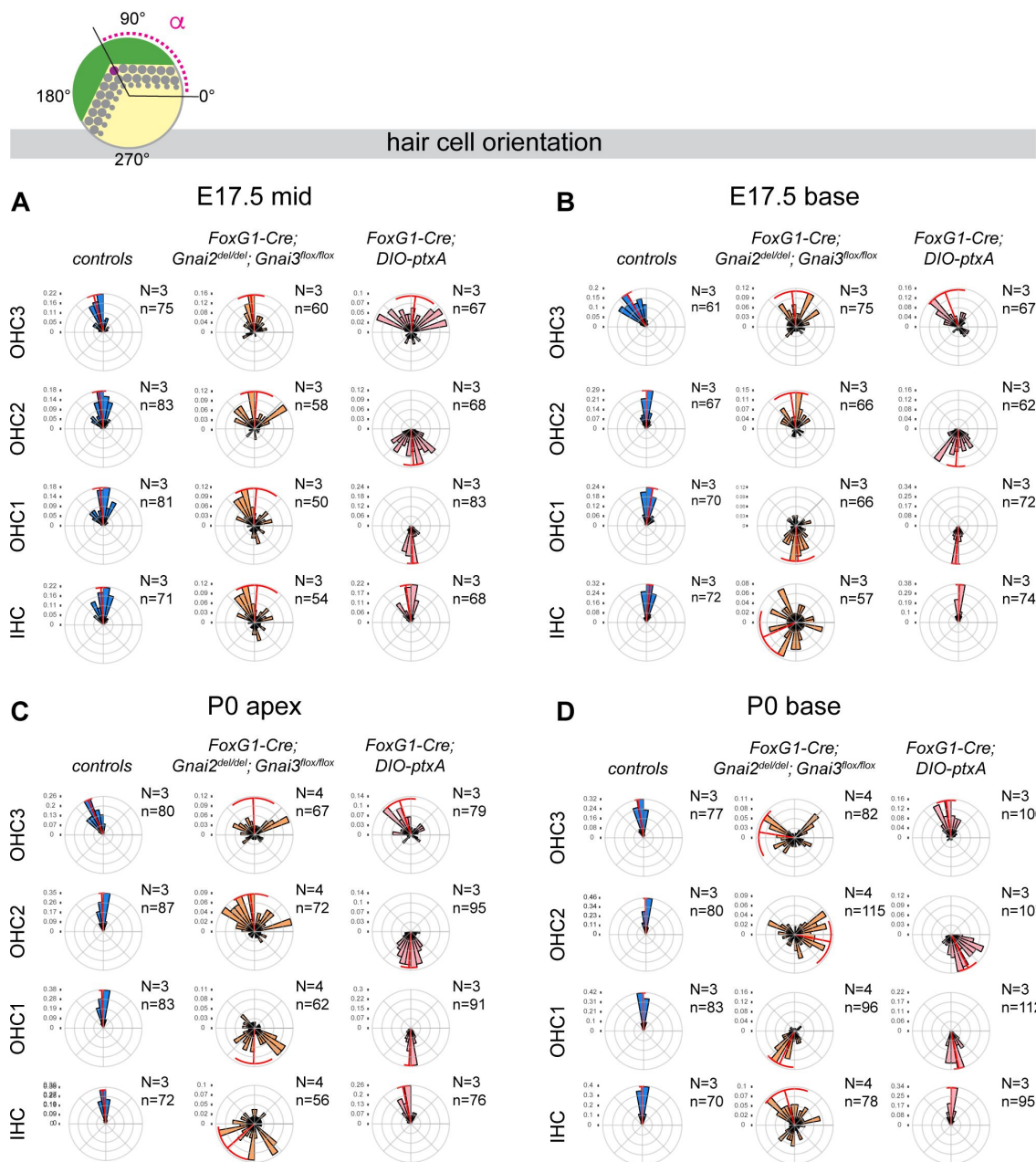


Figure 8.

Loss of GNAI2 and GNAI3 recapitulates hair cell orientation defects observed in *ptxA* mutants.

A-D) Circular histograms show HC orientation (α) based on the position of the basal body (purple dot in top diagram) at the stage and cochlear position indicated. 0° is towards the cochlear base and 90° is lateral. Only HCs with an eccentricity above 0.25 are represented (see Fig. 7 [4](#)). Histograms show frequency distribution (10° bins) and red radial lines and arcs respectively indicate the circular mean and the circular mean deviation. In control cochleae, HCs are tightly oriented laterally (90°) except for OHC3 that show a slight bias towards the cochlear apex (180°). As reported previously, *ptxA* expression induces a reversal of OHC1 and OHC2, and more imprecise lateral orientation of OHC3. This phenotype is progressively recapitulated in time in *Gnai2*; *Gnai3* double mutants (compare least mature E17.5 mid (A) and most mature P0 base (D)). Interestingly, IHCs also show severe misorientation in *Gnai2*; *Gnai3* double mutants, unlike in *ptxA* mutants. n, HC number in N=3-4 animals. Controls for *FoxG1-Cre*; *Gnai2*^{del/del}; *Gnai3*^{flox/flox} are *Gnai2*^{del/+}; *Gnai3*^{flox/+}, *Gnai2*^{del/+}; *Gnai3*^{flox/flox}, *FoxG1-Cre*; *Gnai2*^{del/+}; *Gnai3*^{flox/+} and *FoxG1-Cre*; *Gnai2*^{del/+}; *Gnai3*^{flox/flox}. Histograms for littermate controls of *FoxG1-Cre*; *DIO-ptxA* mutants, as well as data at the P0 cochlear mid position, can be found in Fig. 8 Supp. 1 [4](#). Detailed cohorts and results can be found in the Source Data file.

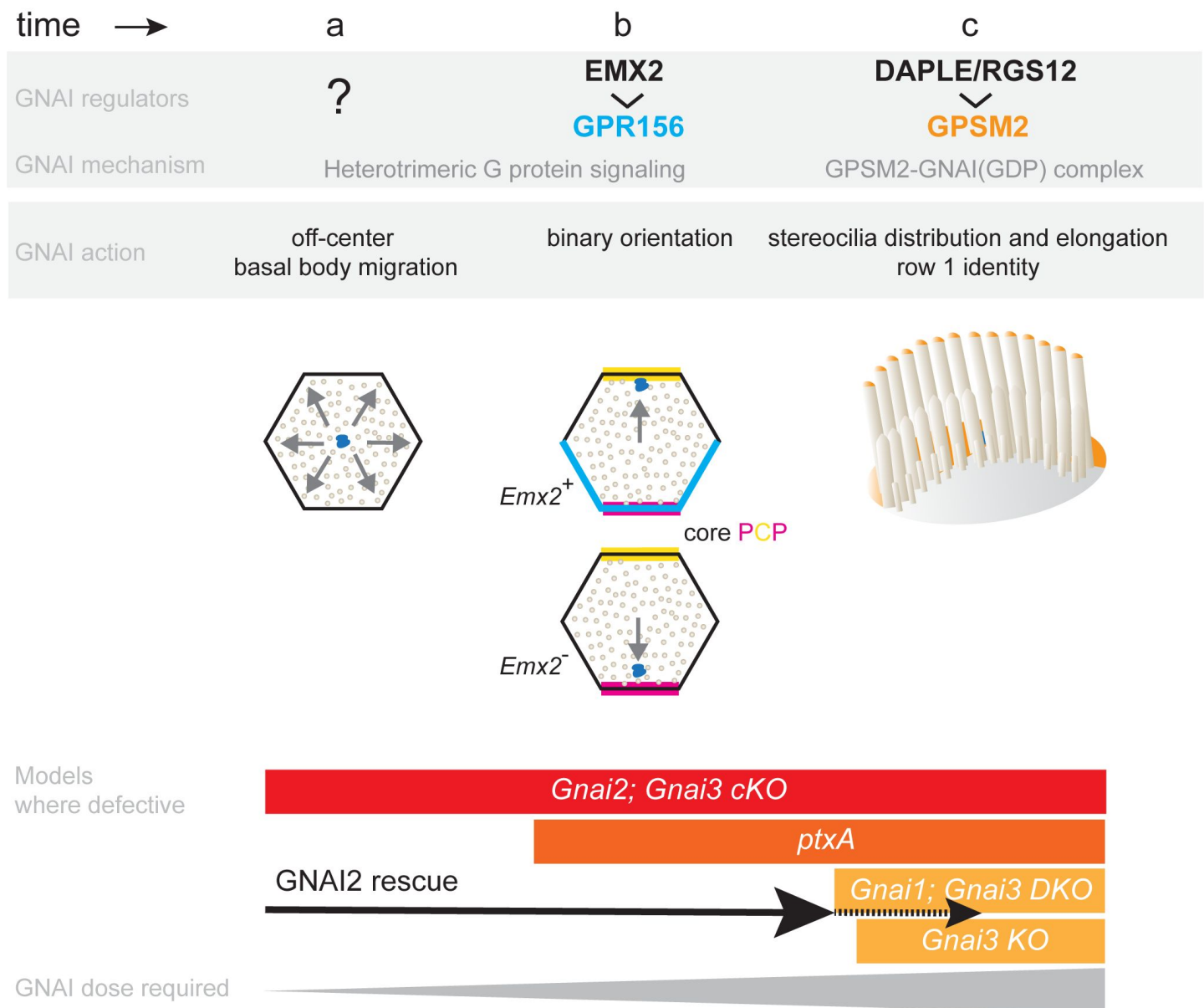


Figure 9.

Summary and model: three roles validated in vivo for GNAI proteins during hair cell polarized morphogenesis.

GNAI proteins are required for the centrifugal migration of the basal body, and thus HC symmetry breaking (a). This activity is distinct from defining binary HC orientation downstream of GPR156 (b), as *Gpr156* mutant HCs have normal basal body positioning and normal hair bundles. Finally, GNAI partners with GPSM2 to shape the hair bundle and elongate stereocilia (c). The specific identity and the dosage of the GNAI proteins required differ for each role. GNAI3 is the principal architect of hair bundle development (c), with GNAI2 playing an important but progressively waning role. In this role, high amounts of GNAI3/GNAI2 are required. A lower dose of any GNAI protein is sufficient for proper HC orientation (b), and a still lower dose can ensure symmetry breaking (a) since this function is intact in the *ptxA* model. We speculate that heterotrimeric signaling underpins basal body migration (a), as it does HC orientation (b). A regulator working with GNAI proteins for symmetry breaking remains to be identified.

Different GNAI/O identity, dosage and timing underlie different functions

Interestingly, besides involving different regulators, each function also involves different dosage and specific identity of the underlying GNAI protein(s) at different times during HC development (Figure 9). For postnatal hair bundle morphogenesis (c), GNAI proteins are sequestered in the GDP state by GPSM2, forming a complex highly enriched at the bare zone and at row 1 stereocilia tips that can be reliably immunodetected (Akturk et al, 2022; Kindt et al, 2021). In these two sub-cellular compartments, the identity of the GNAI proteins at work appears to matter, with GNAI3 playing a necessary and prominent role while GNAI2 is important yet not required. We cannot rule out that GNAI1 participates as well in a minor way because *Gnai1* mutants appear to have more variable stereocilia height than littermate controls (Figure 2C), and we observed more severe stereocilia defects and more elevated ABR thresholds in *Gnai1*; *Gnai3* compared to single *Gnai3* mutants (Figure 2C, E; Figure 3D). The latter observations might instead reflect differences in the genetic background, however. In stark contrast, GNAI2 can fully rescue normal off-center basal body migration (a) and normal HC orientation (b) when both GNAI1 and GNAI3 are constitutively absent (Figure 9). In *FoxG1-Cre*; *Gnai2*; *Gnai3* double mutants, a majority of HCs eventually break symmetry, strongly suggesting that GNAI1 and/or GNAO can participate in the off-center migration mechanism. Finally, even high expression of untagged ptxA in the *DIO-ptxA* model does not affect symmetry-breaking in cochlear HCs, whereas ptxA severely disrupts HC orientation (Figure 9). We thus conclude that i) ptxA downregulates but does not abolish GNAI/O activity in HCs, and ii) the GNAI dosage required to ensure each function is graded (a)<(b)<(c), with probably several GNAI/O proteins able to participate in embryonic functions (a, b). A low dose of GNAI/O dynamically cycling between the GDP and GTP-bound state in heterotrimeric G protein complexes could explain why GNAI proteins are not immunodetected along with GPR156 or other regulators besides GPSM2 (Akturk et al, 2022; Kindt et al, 2021).

A strict dosage- and time-dependence for correct GNAI functions explains why our conditional *Gnai2*; *Gnai3* double mutant model is more severely affected than a comparable conditional *Gnai2*; *Gnai3* model where only hair bundle defects (c) were reported (Beer-Hammer et al, 2018). While Beer-Hammer and colleagues used *FoxG1-Cre* as a driver as we did, combined GNAI2 and GNAI3 inactivation demanded Cre recombination at four floxed loci (*Gnai2*^{flox/flox}; *Gnai3*^{flox/flox}). In contrast, our model demands recombination at two loci only (*Gnai2*^{del/del}; *Gnai3*^{flox/flox}). We thus conclude that our model achieves an earlier and greater loss of GNAI activity. This explanation is supported by extensive perinatal lethality in our model, whereas Beer-Hammer and colleagues could analyze *Gnai2*; *Gnai3* double mutants as adults. Our work thus usefully reconcile all previous reports about GNAI functions, notably showing that inactivating endogenous GNAI proteins does produce early defects (a,b) so far only observed with ptx. We also validate all HC defects observed with ptx as physiologically relevant and specific to GNAI/O function.

Hair cell break of symmetry

Although cochlear HCs expressing ptxA undergo normal symmetry-breaking in vivo (Kindt et al, 2021; Tadenev et al, 2019; Tarchini et al, 2013; Tarchini et al, 2016), a fraction of HCs expressing ptxA in the utricle and saccule have abnormally central basal bodies (Kindt et al, 2021). This suggests that a higher dose of GNAI is required for off-center migration of the basal body in vestibular compared to cochlear HCs, making vestibular HCs more susceptible to ptxA. “Ciliary” proteins are required for ciliogenesis, including kinocilium formation and maintenance, and also play non-ciliary functions (May-Simera et al, 2015; Sipe & Lu, 2011). Inactivation of intraflagellar transport protein IFT88 was reported to result in a central basal body and circular hair bundles in ~10% of OHCs (Jones et al, 2008), but the underlying mechanism was not elucidated. A low proportion of symmetrical HCs were also reported in absence of the CD2 isoform of the protocadherin PCDH15 that forms inter-stereocilia and kinocilium-stereocilia fibrous links

during embryogenesis (Webb et al, 2011 [↗](#)). It remains unclear whether GNAI activity is involved or not in *Ift88* or *Pcdh15* mutant defects, or whether GNAI could be active at the basal body or the kinocilium.

Hair cell orientation

The PCP axis is defined by opposite asymmetric enrichment of the core PCP transmembrane proteins VANGL2 and FZD3/6 and their specific cytosolic partners at HC apical junctions (Deans, 2013 [↗](#); Montcouquiol & Kelley, 2019 [↗](#); Tarchini & Lu, 2019 [↗](#)). Previous work showed that regional *Emx2* expression in HCs reverses how the basal body migrates relative to early-set core PCP landmarks, establishing the line of polarity reversal in the utricle and saccule (Jiang et al, 2017 [↗](#); Tona & Wu, 2020 [↗](#)). EMX2 activates the GPCR GPR156 by triggering its polarized enrichment at the apical HC junction, where downstream GNAI signaling appears to repel the basal body (Figure 9 [↗](#), b) (Kindt et al, 2021 [↗](#)). It is important to note that HCs lacking GPR156 have a normal apical cytoskeleton, including a normally formed hair bundle, and only show orientation defects (Kindt et al, 2021 [↗](#)). This indicates that the centrifugal move of the basal body and the orientation of this move that defines early HC orientation are two distinct molecular mechanisms that both involve GNAI/O proteins (Figure 9 [↗](#); a-b). HC orientation is regulated by core PCP proteins defining an axis of polarization, and by the EMX2>GPR156>GNAI cascade defining binary orientation along that axis. Following the same logic, HCs lacking GPSM2 are not inverted in orientation as in *Emx2*, *Gpr156*, *Gnai2*; *Gnai3* or *ptxA* mutants (Bhonker et al, 2016 [↗](#); Ezan et al, 2013 [↗](#); Tarchini et al, 2013 [↗](#)). This shows that bare zone enrichment of GPSM2-GNAI is a mechanism to shape and polarize the growth of the hair bundle, but not to migrate off-center or orient the basal body. It is worth noting that GPSM2-GNAI is polarized at the HC apical membrane on the side of the off-center basal body, and not at the apical junction where core PCP proteins regulate HC orientation (Siletti et al, 2017 [↗](#); Tarchini et al, 2013 [↗](#)).

While both GPR156 (Greene et al, 2023 [↗](#)) and GPSM2 (Doherty et al, 2012 [↗](#); Walsh et al, 2010 [↗](#)) have been reported as human deafness genes, there is currently no evidence implicating any GNAI/O protein. This is most likely because even GNAI3, which is more specifically required for hair bundle morphogenesis (Figure 9 [↗](#)), plays ubiquitous and critical signaling roles in the context of heterotrimeric protein signaling across many cell types.

Methods

Mouse strains and husbandry

All mouse strains used in this work and strain-related information is summarized in **Supplementary Table 1** [↗](#). All primers used for genotyping are indicated in **Supplementary Table 2** [↗](#) by strain.

Strains from The Jackson Laboratory

Gnai1^{neo}; *Gnai3^{neo}* (*Gnai1^{tm1Lbi}*; *Gnai3^{tm1Lbi}*, MGI: 5560183) (Jiang et al, 2002 [↗](#)) carries a neo cassette in *Gnai1* exon 3 and a neo cassette replacing part of intron 5 and exon 6 in *Gnai3* in the 129S1/SvImJ background. The single *Gnai1^{neo}* and single *Gnai3^{neo}* alleles were segregated from *Gnai1^{neo}*; *Gnai3^{neo}* by breeding with C57BL/6J mice and are consequently on a mixed 129S1/SvImJ; C57BL/6J background. *Gnao1^{neo}* (*Gnao1^{tm1Lbi}*, MGI: 2152685) carries a neo cassette in *Gnao1* exon 6 in the 129S1/SvImJ background (Jiang et al, 2002 [↗](#)). The two *Cre* strains used in this work are *Atoh1-Cre* (*Tg(Atoh1-cre)1Bfri*; MGI: 3775845) expressing Cre in HCs from E14.5 (Matei et al, 2005 [↗](#)) and *FoxG1-Cre* (*Foxg1^{tm1(cre)Skm}*, MGI: 1932522) expressing Cre in the prospective otic vesicle from E8.5 (Hebert & McConnell, 2000 [↗](#)).

Consortium strain

Gnao1^{flox} (*Gnao1^{tm1c(EUCOMM)Hmgu}*) is a derivative of the EUCOMM strain *Gnao1^{tm1a(EUCOMM)Hmgu}* (MGI: 4456727; “KO first, conditional ready”). The *Gnao1^{flox}* conditional allele was produced via FLP-mediated recombination (FLP strain MGI: 4830363) to remove the FRT-flanked *LacZ-neo* cassette, leaving exon 3 floxed. *Gnao1^{flox}* is on a C57BL/6N background.

Constitutive *Gnai2^{del}* inactivation (see **Figure 2 Supp. 1A** [↗](#)).

Gnai2^{del} (*Gnai2^{em1Btar}*; MGI: 6466534) was generated using direct delivery of CRISPR-Cas9 reagents in mouse zygotes via electroporation. The following guide RNAs (gRNA) were used to delete exon 2, exon 3 and part of exon 4 in the C57BL/6J background. Upstream gRNA: CTGCCCTCTGTCCAGGTGC, downstream gRNA: ATGCTTCCTGAAGACCTGTC. The resulting 681 bp deletion encompassed TGCTGGAGAGTCAGGGAAGA...CCTGAAGACCTGTCCGGTGT. The electroporation mixture consisted of the gRNAs with AltR-*Streptococcus pyogenes* Cas9 (SpCas9) V3 (Integrated DNA Technologies #1081059) in embryo-tested TE buffer (pH 7.5). Electroporation of zygotes was performed as described in (Qin et al, 2015 [↗](#)). In order to segregate away potential non-specific mutations, founders were bred for two generations with C57BL/6J animals to generate a N2 heterozygote stock. Because *Gnai2^{del}* homozygotes showed low viability, this strain was used in a mixed C57BL/6J:FVB/J background that improved the proportion of homozygotes (see **Supp. Table 1** [↗](#)).

Conditional *Gnai3^{flox}* inactivation

(see **Figure 2 Supp. 1B** [↗](#)). A plasmid-based donor vector was cloned to flank *Gnai3* exon 2 and 3 with *loxP* sites using the same CRISPR/Cas9 system described above for *Gnai2^{del}*. A fragment carrying a *loxP*, a genomic region including exon 2-3, the restriction site *ClaI*, a second *loxP* and the restriction site *XhoI* was synthesized (Genscript) and cloned between 5' and 3' homology arms amplified by PCR using Gibson assembly. The following gRNAs were used and define the extremities of the floxed region: upstream, AGTCCACAAAATTCCCATT, TAGGGGATATAGATCCAAAT, downstream, TTCCAGGACTCTGCATGCGT, TACCGACGCATGCAGAGTCC. The gene editing reagents (gRNAs, donor vector, SpCas9) were microinjected in zygotes as described in (Qin et al, 2015 [↗](#)). To confirm insertion at the *Gnai3* locus, we performed long-range PCRs with a genomic primer located outside the homology arm on each side: 5' long-range: F(external)_TACTGAGATGAGAGACTGAGGG and R (internal)_TGGCTGACATCCTTTGATGGAC. The 3070 bp product was digested with *XhoI*, producing 2,551 + 519 bp fragments upon donor insertion (flox allele). 3' long-range: F (internal)_TGAAAGGTAAAGGCAACGTGAG and R (external)_TGTGAGACAGGGTCTCTCTTG. The 2,966 bp product was digested with *XhoI*, producing 2,000 + 966 bp fragments upon donor insertion (flox allele). In order to segregate away potential non-specific mutations, founders were bred for two generations with C57BL/6J animals to generate a N2 heterozygote stock.

Pertussis toxin catalytic subunit (ptxA)-expressing strains

(see **Figure 2 Supp. 1C-D** [↗](#)). The *LSL-myc:ptxA* strain at the *ROSA26* (*R26*) locus was described previously (*Gt(ROSA)26Sor^{em1(ptxA)Btar}*; MGI: 6163665) (Tarchini et al, 2016 [↗](#)). The new *CAG-DIO-ptxA* strain line (see **Figure 2 Supp. 1C** [↗](#)) was generated at the *R26* locus using the Bxb1 attP(GT) integrase technology and *C57BL/6J-Gt(ROSA)26Sor^{Mvw}* (MGI: 6757188) as the host strain (Low et al, 2022 [↗](#)). The donor vector consisted in a CAG promoter followed by the flipped *ptxA* coding sequence flanked by double inverted *lox* sites (*loxP* and *lox2272*; **Figure 2 Supp. 1C** [↗](#)) and a bGHpA sequence. In order to exclude the prokaryotic vector backbone, the donor vector was prepared as a minicircle using the MC-Easy Minicircle Production kit (SystemBio, MN920A-1). Briefly, the donor plasmid insert was cloned into a Minicircle Cloning Vector using PhiC31 integrase before transformation into the ZYCY10P3S2T *E. coli* minicircle producer strain, which

after induction with arabinose, results in the generation of the donor minicircle. To eliminate parental plasmid contamination, a restriction digest was performed, followed by MC-safe DNase treatment. The minicircle DNA was then purified by phenol-chloroform extraction and reconstituted in microinjection buffer (10 mM Tris; 0.1 mM EDTA pH7.5). The *CAG-DIO-ptxA* strain was generated by pronuclear microinjection of the Bxb1 Integration reagents directly into zygotes of the host strain. These reagents included the Bxb1 mRNA (Trilink) at 100ng/μl, RNasin (Promega) at 0.2U/μl and the donor minicircle DNA at 10ng/μl combined in microinjection buffer. To confirm successful integration of the 3,212bp transgene, as well as identify random transgenics, the initial screening was performed using a four-PCR strategy. The In/Out Left and Right (IOL, IOR) PCRs, which bridge the recombined attachment sites, each contain one primer specific to the *R26* locus and a second primer designed against the inserted sequence. The Transgene (TG) PCR amplifies a non-genomic sequence in the insert, and the Off-Target Integration (OTI) PCR was designed to detect non-recombined minicircle integration, presumably outside of the *R26* locus.

IOL PCR (F1/R1), F1: GTCGCTCTGAGTTGTTATCAGT, R1: GCCAAGTAGGAAAGTCCCATAA (720 bp, WT=no band). IOR PCR (F2/R2), F2: GGTGATGCCGTTGTGATAGA, R2: TGTGGGAAGTCTTGTCCCTCCAAT (1,013 bp, WT=no band). TG PCR (F2/R3) F2: 5'-GGTGATGCCGTTGTGATAGA, R3: CCACTTCATCGGCTACATCTAC (214 bp, WT=no band). OTI PCR (F3/R1) F3: GGGAGGATTGGGAAGACAATAG, R1: GCCAAGTAGGAAAGTCCCATAA (578 bp, WT=no band). 33 mice were born following 6 transfers totaling 117 injected embryos (28% survival), and 1/33 (3%) was identified as a founder and crossed to C57BL6/J to generate N1 offspring. The In/Out PCR confirmed that a copy of the transgene was inserted at the *R26* locus, but the OTI strategy also gave a product, even after breeding for multiple generations. As breeding would have segregated a separate, random integration of the transgene, we performed Nanopore-based Cas9-targeted sequencing at the *R26* locus (Gilpatrick et al, 2020 [DOI](#); Low et al, 2022 [DOI](#)). This revealed that, as seen in some cases previously (Low et al, 2022 [DOI](#)), tandem insertions had occurred in this founder. Specifically, 3 consecutive copies of the *CAG-DIO-ptxA* transgene were inserted at the *R26* locus in this strain. This did not impact specific Cre-based expression of *ptxA* because phenotypes observed in this new strain were comparable to the previous *LSL-myc:ptxA* strain (**Figure 6 Supp.1 B-D** [DOI](#)).

Experimental animals in the study ranged in age between E17.5 and P29 as indicated in each figure. Male and females were systematically included but sex was not tracked except for Auditory Brainstem Recordings because there is no evidence that sex influences HC orientation or hair bundle morphogenesis. Animals were maintained under standard housing conditions (14h light / 10h dark cycle, ambient temperature and normal humidity). All animal work was reviewed for compliance and approved by the Animal Care and Use Committee of The Jackson Laboratory (Animal Use Summary AUS #14012).

Scanning Electron Microscopy (SEM)

Temporal bones were isolated, punctured at the cochlear apex, and fixed by immersion for at least one overnight at 4°C in 2.5% glutaraldehyde (Electron Microscopy Sciences; 16200) and 4% paraformaldehyde (Electron Microscopy Sciences; 15710) in 1mM MgCl₂, 0.1M Sodium Cacodylate, 20mM CaCl₂. Samples were rinsed and decalcified overnight in 4% EDTA. The auditory epithelium was then dissected into 3 pieces (cochlear base, mid, and apex) before progressive dehydration in an ethanol series (30-50-70-80-90-100%, at least 20 minutes per step) and chemical drying with hexamethyldisilazane (HMDS; Electron Microscopy Sciences 50-243-18). Dry samples were mounted on aluminum stubs using double-sided carbon tape and sputter-coated with gold-palladium before imaging on a Hitachi 3000N VP electronic microscope at 20kV.

Immunofluorescence and antibodies

For embryonic and postnatal stages, temporal bones were either immediately dissected to expose the sensory epithelium and fixed in paraformaldehyde (PFA 4%; Electron Microscopy Sciences; 15710) for 1h at 4°C. After fixation, the tectorial membrane was removed, and samples were

permeabilized and blocked in PBS with 0.5% Triton-X100 and bovine serum albumin (1%) for at least at 1 hour at room temperature. For adult stages, temporal bones were isolated and punctured at the cochlear apex to facilitate access for the fixative. Samples were then immersion-fixed in PFA 4% for 1 hour at 4°C, rinsed in PBS, and incubated overnight in 4% EDTA for decalcification. Cochleae were next dissected in 3 pieces (cochlear base, mid, and apex), before permeabilization and blocking as described for earlier stages. Primary and secondary antibodies were incubated overnight at 4°C in PBS with 0.025% sodium azide. Fluorescent dye-conjugated phalloidin was added to secondary antibodies. Samples were washed 3 times in PBS + 0.05% Triton-X100 after each antibody incubation and post-fixed in PFA 4% for at least 1 hour at room temperature. Samples were then mounted flat on microscopy slides (Denville M102) using Mowiol as mounting medium (Calbiochem/MilliporeSigma 4759041), either directly under a 18×18mm #1.5 coverglass (VWR 48366-045) (postnatal cochleae) or using one layer of office tape as a spacer (adult cochleae). Mowiol (10% w/v) was prepared in 25%(w/v) glycerol and 0.1M Tris-Cl pH8.5. Primary antibodies used were:

Rabbit anti-GNAI2, pt"GNAI2" (Proteintech, 11136-1-AP); the antigen is the full human GNAI2 protein, and GNAI1 is a closer paralog to GNAI2 compared to GNAI3. Rabbit anti-GNAI3, scbt"GNAI3" (Santa Cruz Biotechnology, sc-262); the C-terminal rat antigen was not disclosed but the company indicated that this product detects preferentially GNAI3>GNAI1>GNAI2. Rabbit anti-GNAO (Proteintech, 12635-1-AP) Mouse anti-acetylated alpha tubulin (Santa Cruz Biotechnology scbt-23950) Rabbit anti-GPSM2 (Sigma, A41537) Goat anti-GPSM2 (ThermoFisher, PA5-18646) Rabbit anti-Pericentrin/PCNT (Biolegend/Covance, PRB-432C) Rat anti-ZO1 (Developmental Studies Hybridoma Bank, R26.4C)

Secondary antibodies from ThermoFisher Scientific were raised in donkey and conjugated to Alexa Fluor (AF) 488, 555, or 647 (donkey anti-rabbit AF555 (A-31572), AF647 (A-31573), donkey anti-mouse AF647 (A-31571), donkey anti-rat AF488 (A-21208), donkey anti-goat AF555 (A-21432), AF647 (A-21447)). Fluorescent conjugated phalloidins used to reveal F-actin were from ThermoFisher Scientific: AF488 (A12379); AF555 (A34005) and Sigma-Aldrich: FITC (P5282).

Sample cohorts, image acquisition and analysis

All quantifications included at least three animals per genotype. All graphs or their legends indicate the animal cohort size (N) as well as the number of HC or stereocilia (n) analyzed. Further details can be found in a single Supplementary Source Data file where tabs are referencing figure panels in order. When an experimental outcome was not quantified, at least 3 mutant and 3 control littermates encompassing two or more litters were analyzed, and figure panels illustrate a representative outcome observed in all samples of the same genotype.

Confocal images were captured with a LSM800 line scanning confocal microscope, a 63x NA1.4 oil objective, the Airyscan detector in confocal mode (except in **Figure 5 Supp.1 D, E** where the Airyscan detector was used in Airyscan mode) and the Zen 2.3 or Zen 2.6 software (Carl Zeiss AG). Raw Airyscan images in **Figure 5 Supp.1 D, E** were processed in Zen 2.6 selecting for automatic strength. Unless stated otherwise in the legends, images show a single optical z plane. To quantify HCs eccentricity (**Figure 7**), images were captured with a Leica DM5500B widefield microscope, a 63x oil objective, a Hamamatsu ORCA-Flash4.0 sCMOS camera and the Leica Application Suite (LasX) software (Leica Microsystems). All images in the same experiment were acquired using the same laser intensity and gain, and were then processed in Adobe Photoshop (CC2020) where the same image treatment was applied across conditions.

To measure stereocilia length and width in IHCs (**Figure 2C-D**), SEM samples were imaged laterally with a 10,000x magnification and an appropriate tilt (from 0 to 30°) to bring stereocilia parallel to the imaging plane and minimize parallax. To quantify the number of rows in adult IHCs and the number of stereocilia in their first row (**Figure 2E, F**), SEM samples were imaged

medially with a 5,000x magnification. To measure the length of OHC hair bundle wings (**Figure 2G** [↗](#), **Figure 2 Supp. 1G** [↗](#)), OHCs were imaged from the top at 5,000x. Stereocilia width was measured at half-length and all measurements were done with the straight-line tool in Fiji.

To quantify GNAI signal intensity in *Gnai1^{neo}*; *Gnai3^{neo}* mutants (**Figure 5C** [↗](#), **Figure 5 Supp. 1A** [↗](#)), Z-stack series were acquired at the one-turn cochlear position. A single Z slice was chosen at the bare zone level, another one at the stereocilia tip level, each based on strongest signal in that compartment. The vertex of the V-shaped hair bundle was used to divide the HC apical surface in two equal halves. In each half (left and right), regions of interest (ROIs) were drawn using the polygon selection tool in Fiji to encompass all signals in that apical compartment, and mean gray values were measured. For each image, background signal was measured and averaged, and subtracted from all measurements in the same image.

To quantify surface area of the bare zone or total surface area in half-OHC as well as the length of half hair bundles (**Figure 5G** [↗](#), **Figure 5 Supp. 1C** [↗](#); **Figure 6D**, **H** [↗](#)), Z-stack series were acquired at the cochlear base and a single Z slice was selected at apical junction level using ZO1 (**Figure 5G** [↗](#), **Figure 5 Supp. 1C** [↗](#)) or F-actin (**Figure 6D** [↗](#), **H**) as reference. To measure total apical surface area and bundle length in half-OHCs, the pericentrin-stained basal body was used to divide the apical surface in two halves (**Figure 5G** [↗](#), **Figure 5 Supp. 1C** [↗](#)). The ZO1-positive cell outline along with the dividing line at the basal body level were used as reference to measure surface area with the polygon selection tool in Fiji. To quantify the bare zone surface area (**Figure 6D** [↗](#), **H**), a ROI was drawn around the total apical surface lacking phalloidin (F-actin) signals. The length of the F-actin stained hair bundle was measured using the straight-line tool in Fiji.

To determine HC eccentricity (**Figure 7** [↗](#)), the geometrical center of the HC apical surface was determined as the intersection of two orthogonal lines representing the maximal diameter of the cell along the cochlear longitudinal and radial axes. A vector (BB) was drawn from the center to the pericentrin-labeled basal body, and eccentricity was calculated as the ratio of BB length over the cell radius (r) along the same trajectory using F-actin-labeled apical junction as landmark (see **Figure 7** [↗](#)). To determine cell orientation (**Figure 8** [↗](#) and **Figure 8 Supp. 1** [↗](#)), the angle (α) separating the longitudinal axis of the organ of Corti from the BB vector was measured with the angle tool in Fiji (see **Figure 8** [↗](#)). Both right and left cochleae were used and angles were measured so that 0° pointed toward the cochlear base and 90° towards the cochlear periphery (lateral). Eccentricity and angles were measured at the positions indicated (base ~20%, mid ~50%, apex ~80% of the cochlear length starting from the base).

Auditory Brainstem Response (ABR) tests

All tests were performed in a sound-attenuating chamber, and body temperature of the anesthetized animals was maintained at 37°C using a heating pad (FHC Inc.). Animals from all strains except *Atoh1-Cre*; *Gnao1^{fllox}* (**Figure 3 Supp. 1B** [↗](#), see below) were anesthetized with a mix of ketamine and xylazine (1 mg and 0.8 mg per 10g of body weight, respectively) and tested using the RZ6 Multi-I/O Processor System coupled to the RA4PA 4-channel Medusa Amplifier (Tucker-Davis Technologies). ABRs were recorded after binaural stimulation in an open field by tone bursts at 8, 16, 32, and 40 kHz generated at 21 stimuli/second, and a waveform for each frequency/dB level was produced by averaging the responses from 512 stimuli. Subdermal needles were used as electrodes, the active electrode inserted at the cranial vertex, the reference electrode under the left ear and the ground electrode at the right thigh.

Atoh1-Cre; *Gnao1^{fllox}* animals (**Figure 3 Supp. 1B** [↗](#)) were anesthetized with tribromoethanol (2.5mg per 10g of body weight) and tested with the Smart EP evoked potential system from Intelligent Hearing Systems (IHS). ABR were recorded after single ear stimulation (right ear), using ear tubes speakers (ER3C insert earphones, IHS) delivering tone bursts at 8, 16, and 32 kHz generated at 40 stimuli/second. Electrodes were positioned as previously described except for the ground electrode that was placed at the base of the tail. ABR thresholds were obtained for each

frequency by reducing the sound pressure level (SPL) by 5 decibels (dB) between 90 and 20 dB to identify the lowest level at which an ABR waveform could be recognized. We compared waveforms by simultaneously displaying 3 or more dB levels on screen at the same time.

Statistical Analyses

All data were plotted in Prism 9 (GraphPad), except for circular diagrams of HC orientation (**Figure 8** [↗](#); **Figure 8 Supp. 1** [↗](#)) that were generated with R (4.2.2) and Rstudio (2022.12.0+353).

All data except for angles in circular diagrams (**Figure 8** [↗](#) and **Figure 8 Supp. 1** [↗](#)) were plotted individually. Distribution was framed with 25-75% whisker boxes where exterior lines show the minimum and maximum, the middle line represents the median, and + represents the mean. Potential differences in data distribution between genotypes were tested for significance using Mann-Whitney U test (non-parametric unpaired test), except for ABR thresholds (**Figure 3** [↗](#) and **Figure 3 Supp. 1** [↗](#)) where a 2-way ANOVA with Sidak's multiple comparison post-hoc test was used. OHC half-bundle lengths (**Figure 2G** [↗](#), **Figure 2 Supp. 1G** [↗](#)) were plotted as paired left and right values in the same HC and a potential difference in data variance between genotypes was tested using a F-test. GNAI signal intensity at the OHC bare zone and stereocilia tips (**Figure 5C** [↗](#), **Figure 5 Supp. 1A** [↗](#)) as well as OHC surface area and half-bundle length (**Figure 5G** [↗](#), **Figure 5 Supp. 1C** [↗](#)) were plotted and a simple linear regression curve was calculated and drawn for each pair of datasets. A correlation between variables was addressed with Pearson's correlation test. Exact p-values were indicated on each graph when non-significant ($p > 0.05$) and otherwise summarized as follows: $p < 0.0001$ ****, $p < 0.001$ ***, $p < 0.01$ **, $p < 0.05$. Details of statistical results for all figures can be found in the Supplementary Data file.

Angle frequency distribution (**Figure 8** [↗](#) and **Figure 8 Supp. 1** [↗](#)) was plotted in circular diagrams using the R package `dyplr` and the `coord_polar` function of the `ggplot2` package to organize data and produce the graphs, respectively. The angle formed by the red line indicates the circular mean and the length of the arc at the end of the red line indicates the mean circular deviation. Both values were obtained using the `colstats` function in the R circular package. All scripts used in this work will be posted on github.

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Author contribution

B.T. conceived the study and designed most experiments. A.J. and A.L.T. designed, performed and analyzed experiments. M.D. provided technical help with mouse colony maintenance, sample processing and genotyping. B. K. wrote the R script to generate the circular histograms. B.E.L. and M.V.W. helped generate the *DIO-ptxA* strain using BxB1 recombination technology. B.T. supervised the work. B.T. and A.J. wrote the manuscript. B.T. secured funding.

Competeting interests

The authors declare no competing interest.

Data availability

The data in all graphical representations are included in a single Source Data file. Original material used for quantifications and to build figures will be deposited in a publicly accessible repository.

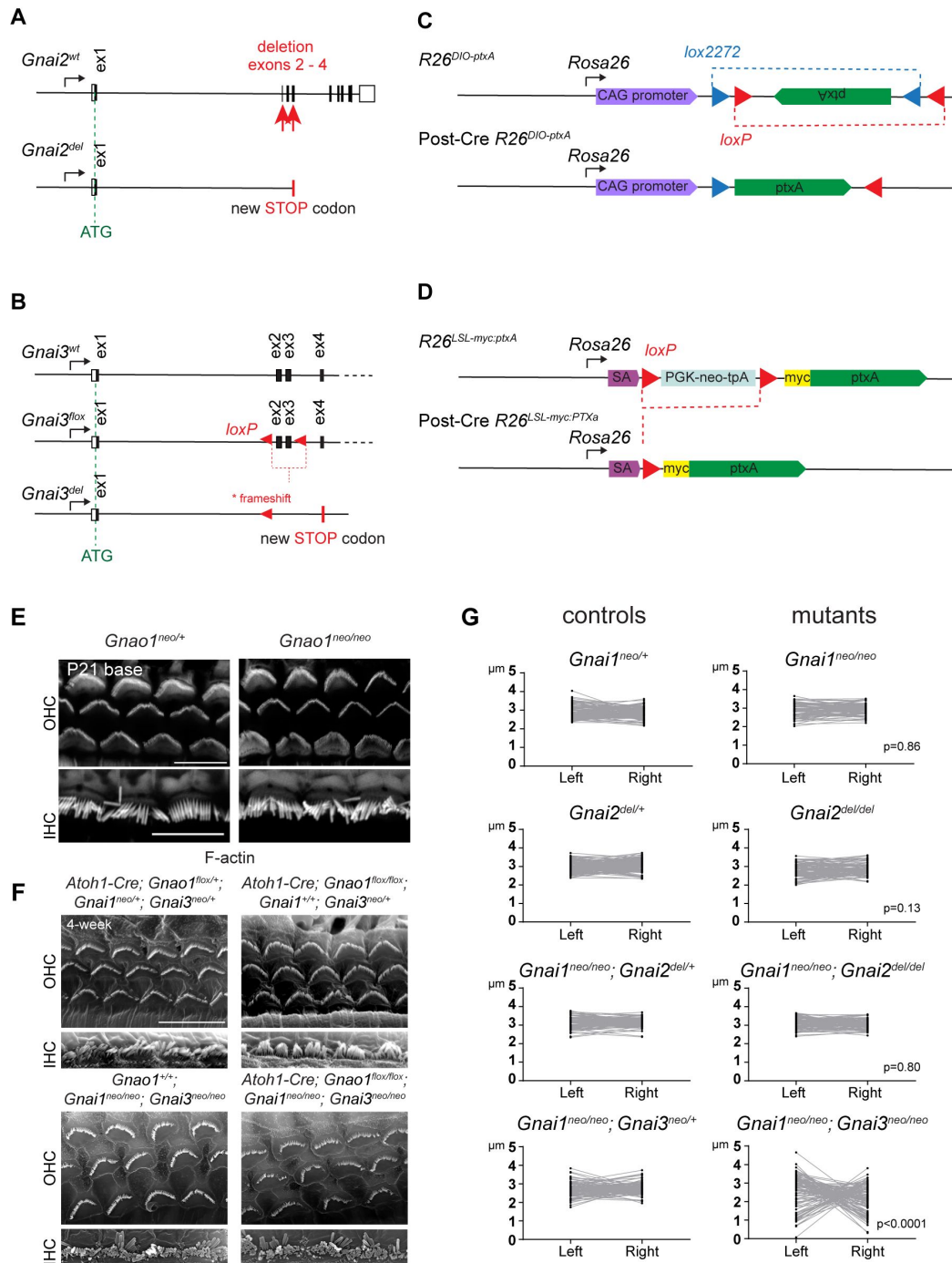


Figure 2 Supplement 1.

A-D) New mouse strains generated in this study are *Gnai2*^{del} (A), *Gnai3*^{lox} (B) and *R26*^{DIO-ptxA} (C; see Methods for details). *R26*^{LSL-myc:ptxA} was published previously and is illustrated for comparison as it is used in the study as well (D). E-F) Loss of GNAO has no obvious impact on apical HC morphology or HC orientation. Representative confocal images of phalloidin-stained *Gnao1* constitutive mutants at P21 (E; *Gnao1*^{neo}) and scanning electron microscopy images of conditional *Gnao1* inactivation in the *Gnai1*; *Gnai3* double mutant background (F; *Gnao1*^{lox}). In F, top panels compare control (left) and conditional *Gnao1* inactivation (right) when GNAI1 and GNAI3 functions are preserved. Bottom panels compare control (left) and conditional *Gnao1* inactivation (right) when GNAI1 and GNAI3 are inactivated. Note how HC-specific inactivation of GNAO using the *Atoh1-Cre* driver does not obviously enhance defects observed in *Gnai1*; *Gnai3* double mutants (F, bottom). G) Graphs showing the length of the left and right wing of 3-week-old OHC hair bundles paired by cell. Graphs for control littermates that were omitted in Fig. 2G are added here (left) next to repeated mutant graphs (right). p-values are for a F-test of variance of pooled left and right wing lengths compared to littermate controls. At least 3 animals and 88 OHCs are represented per genotype. Only *Gnai3*^{neo} (Fig. 2G) and *Gnai1*^{neo}; *Gnai3*^{neo} mutants show truncated hair bundles and a significant p value (p<0.05).

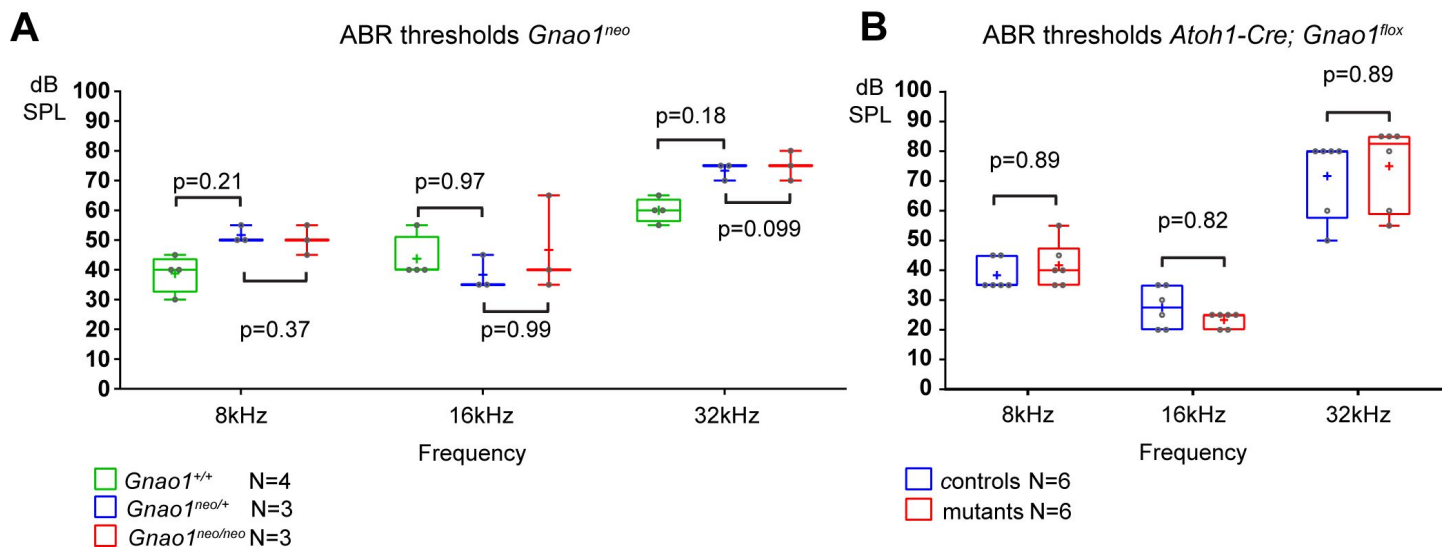


Figure 3 Supplement 1.

A-B) ABR thresholds at 8, 16, 32 kHz for constitutive *Gnao1* (A) and conditional *Atoh1-Cre; Gnao1^{lox/flox}* (B) mutants tested between P20 and P25. Boxplots are framed with 25-75% whisker boxes where exterior lines show the minimum and maximum values, the middle line represents the median, and + represent the mean. N indicates the number of animals tested. In (B), because the original animal cohort was established to test whether the loss of GNAO could enhance defects in *Gnai1; Gnai3* double mutants (see **Fig. 2 Supp. 1E-F**), controls include the following genotypes: *Atoh1-Cre; Gnao1^{lox/+}*, *Gnao1^{lox/flox}*, *Gnai1^{neo/+}*, *Gnai3^{neo/+}*, *Gnai1^{neo/+}*, *Atoh1-Cre; Gnao1^{lox/+}*, *Gnai1^{neo/neo}*, *Gnai3^{neo/+}* and *Gnai1^{neo/neo}*, *Gnai3^{neo/+}* where other alleles are wild-type. Mutants include the following: *Atoh1-Cre; Gnao1^{lox/flox}*, *Gnai3^{neo/+}* and *Atoh1-Cre; Gnao1^{lox/flox}*, *Gnai1^{neo/neo}*, *Gnai3^{neo/+}* where other alleles are wild-type. Cohort details can be found in the Source Data file. Two-way ANOVA with Sidak's multiple comparison. non-significant p-values are indicated. kHz, kilohertz, dB SPL, decibel sound pressure level.

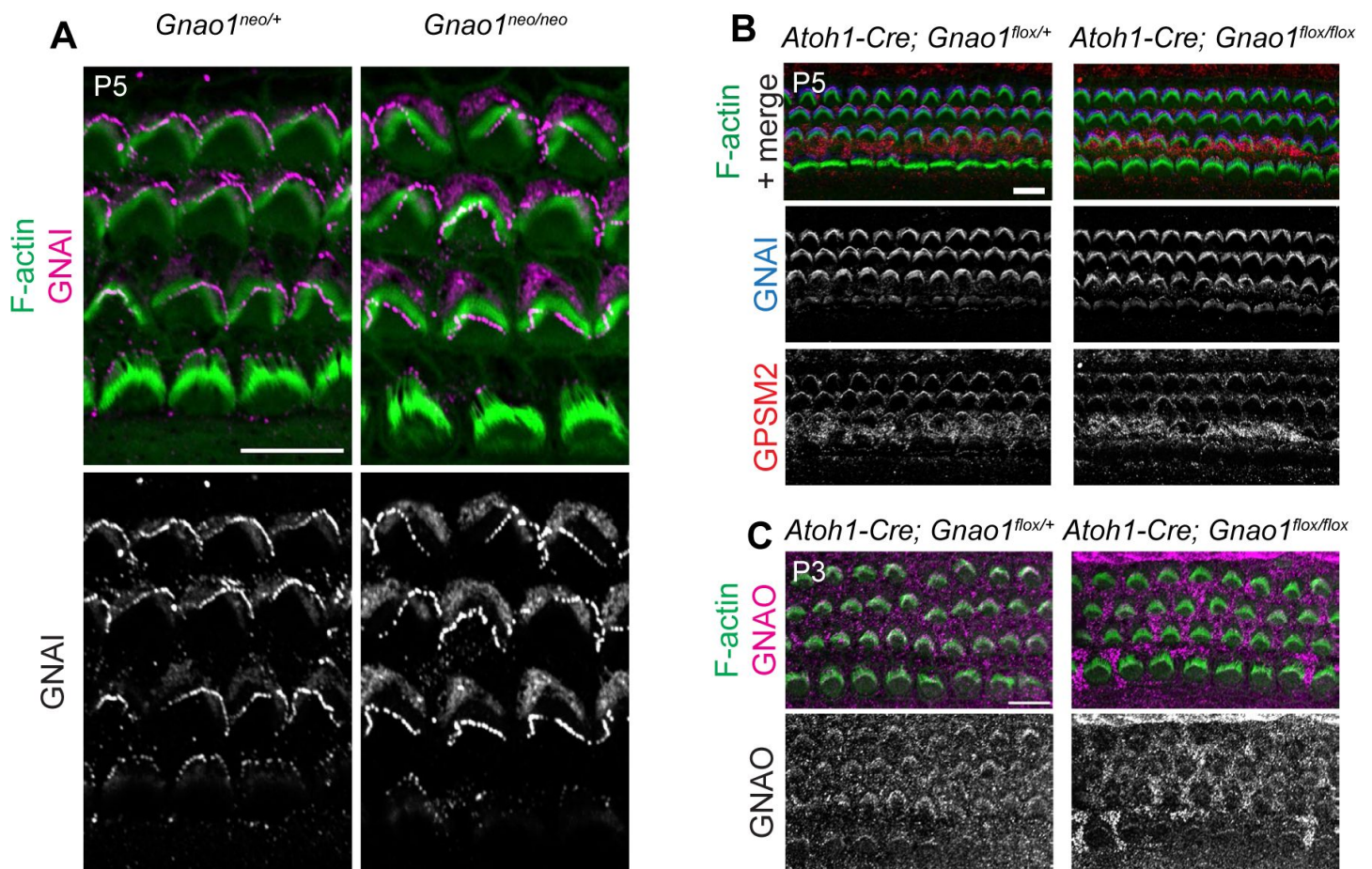


Figure 4 Supplement 1.

GNAI protein distribution is undisturbed in *Gnao1* mutants and no evidence for specific GNAO enrichment at the hair cell apex. A-B) GNAI (scbt “GNAI3” antibody; see **Fig. 4** and Methods) immunolabeling in P5 *Gnao1* constitutive (A) and *Atoh1-Cre; Gnao1^{flox}* conditional (B) mutants at the cochlear base. GNAI and GPSM2 (B) distribution at the bare zone and stereocilia tips is unchanged in absence of GNAO. C) GNAO immunolabeling at the P3 cochlear mid produces signals that are unspecific since they appear unchanged in conditional *Gnao1* mutants. Scale bars are 10µm.

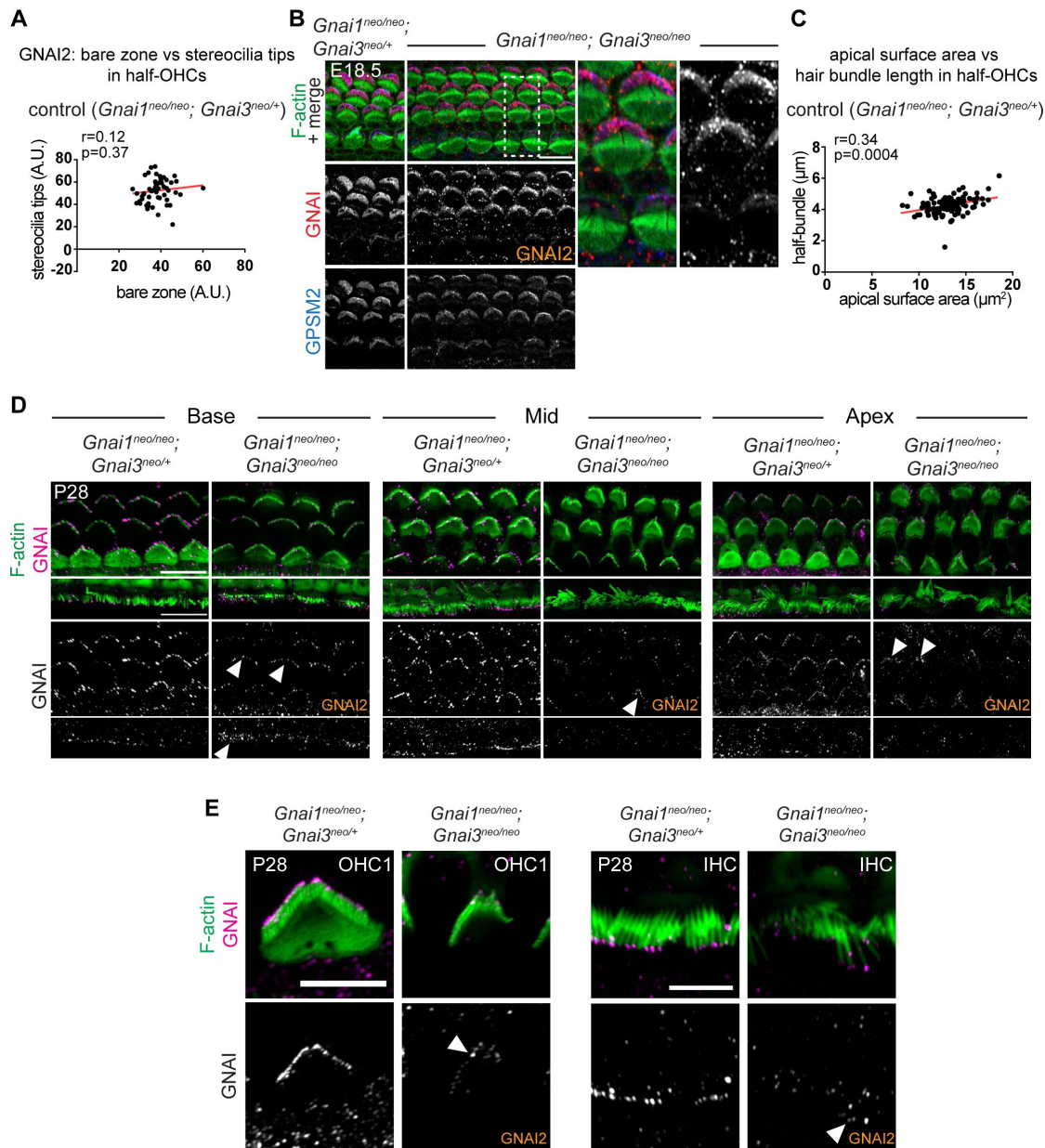


Figure 5 Supplement 1.

GNAI2 fully rescues loss of GNAI3 at embryonic stages and is still detected in low amounts at stereocilia tips in adult hair cells lacking GNAI3.

A) Control correlation plot for *Gnai1*; *Gnai3* double mutants in **Fig. 5C**. GNAI signal intensity at the bare zone and stereocilia tips in control half-HCs at the P2 cochlear base. N=3 animals, n=40 OHCs, Pearson correlation with best fit (red line). The graph shows relatively uniform GNAI enrichment in each sub-cellular compartment, unlike in *Gnai1*; *Gnai3* double mutants (**Fig. 5C**). **B)** GNAI (pt “GNAI2” antibody; see **Fig. 4**) and GPSM2 co-immunolabeling at the E18.5 cochlear base. As sole remaining GNAI, GNAI2 can still encompass the entire bare zone in *Gnai1*; *Gnai3* double mutants and no obvious hair bundle defects are observed, unlike at P0 (**Fig. 5A-B**). The boxed region is magnified to the right. **C)** Control correlation plot for *Gnai1*; *Gnai3* double mutants in **Fig. 5G**. The position of the basal body was used to determine the vertex (middle) of the hair bundle and to draw a radial line separating each OHC into two halves. The length of each hair bundle wing (y axis) is graphed in relation to the corresponding apical surface area (x axis) in the same half-OHC. The plot shows relatively uniform wing lengths and half apical areas in littermate controls, unlike in *Gnai1*; *Gnai3* double mutants (**Fig. 5G**). N=3 animals, n=52 OHCs, Pearson correlation with best fit (red line). **D-E)** GNAI (pt “GNAI2” antibody; see **Fig. 4**) immunolabeling in P28 adults. As sole remaining GNAI, GNAI2 can still be detected at low levels at stereocilia tips in *Gnai1*; *Gnai3* double mutants at the cochlear base, mid and apex (arrowheads). **E)** Higher magnifications views of a single OHC1 (left) and IHC (right). A.U., arbitrary unit. Scale bars are 10µm (B, D) and 5 µm (E).

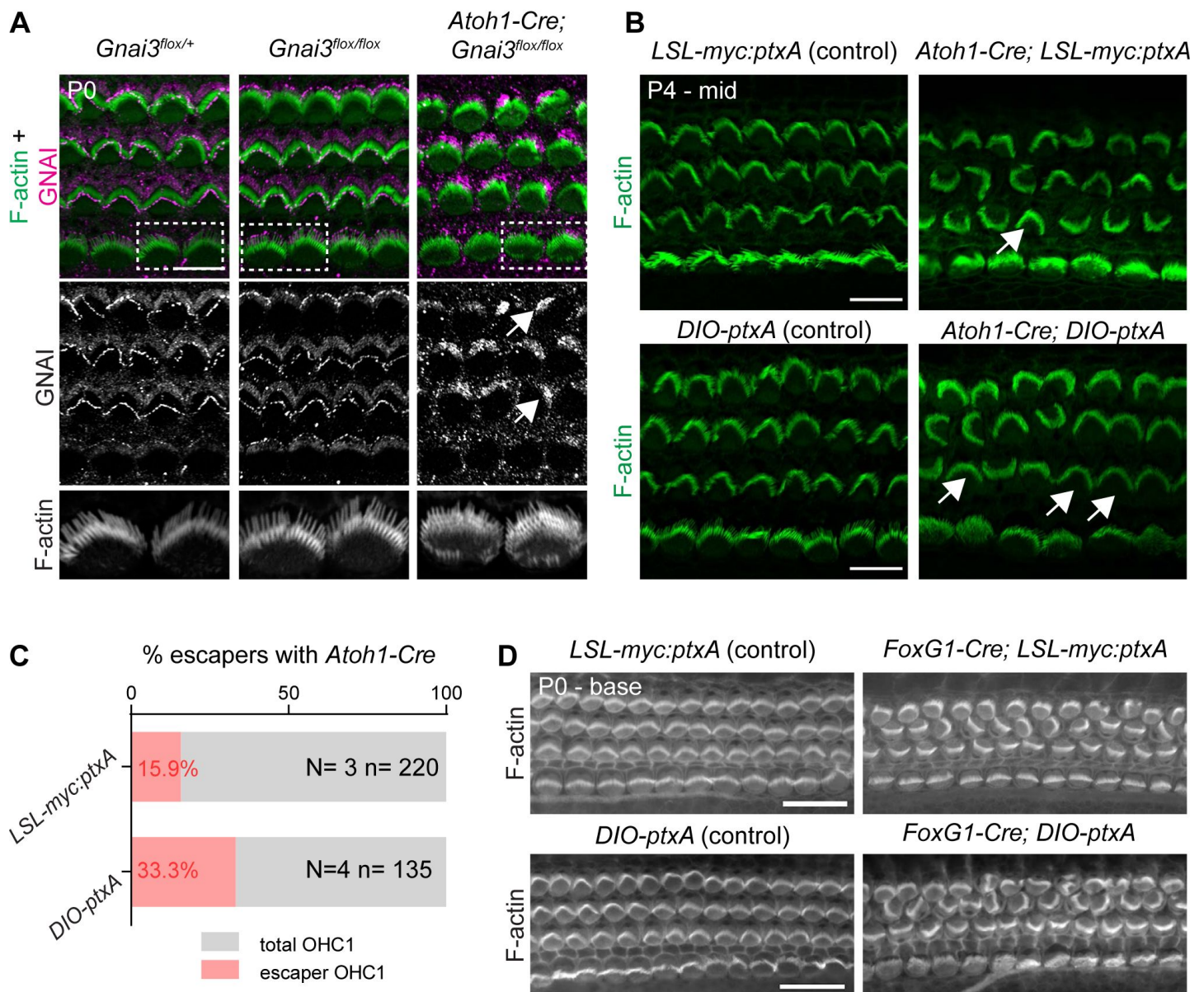


Figure 6 Supplement 1.

Validation of the *Gnai3^{flox}* and *DIO-ptxA* mouse strains.

A) GNAI (pt “GNAI2” antibody; see Fig. 4) immunolabeling at the P0 cochlear base. Note how in absence of Cre recombinase, *Gnai3^{flox/flox}* HCs have normal hair bundles and normal GNAI enrichment at the bare zone and stereocilia tips. In contrast, breeding in *Atoh1-Cre* produces incomplete GNAI enrichment (arrows; compare with Fig. 4D) and shortened stereocilia typical of constitutive *Gnai3* mutants. Boxed IHC regions are magnified below. **B-D)** Comparison of the two *ptxA* strains used in this study (see also Fig. 2 Supp. 1C-D). **B)** PtxA expression with the *Atoh1-Cre* driver produces comparable P4 apical HC defects in the *LSL-myc:ptxA* and *DIO-ptxA* strains. “Escaper” OHC1 that are not inverted in orientation (arrows) likely do not express Cre, or did not undergo recombination. **C)** When *Atoh1-Cre* induces *ptxA* expression in post-mitotic HCs, the number of escaper OHC1 is higher in the *DIO-ptxA* strain. This is likely because *lox*-based genomic deletions (LSL) are favored over genomic inversions (DIO; see Fig. 2 Supp. 1C-D). **D)** In contrast, an earlier Cre driver (*FoxG1-Cre*) shows virtually no escapers in either strain. N and n indicate the number of animals and OHC1 analyzed, respectively. All *ptxA* samples in the study are heterozygotes (*R26^{LSL-myc:ptxA/+}* or *R26^{DIO-ptxA/+}*). Scale bars are 10µm (A, B), 20µm (D).

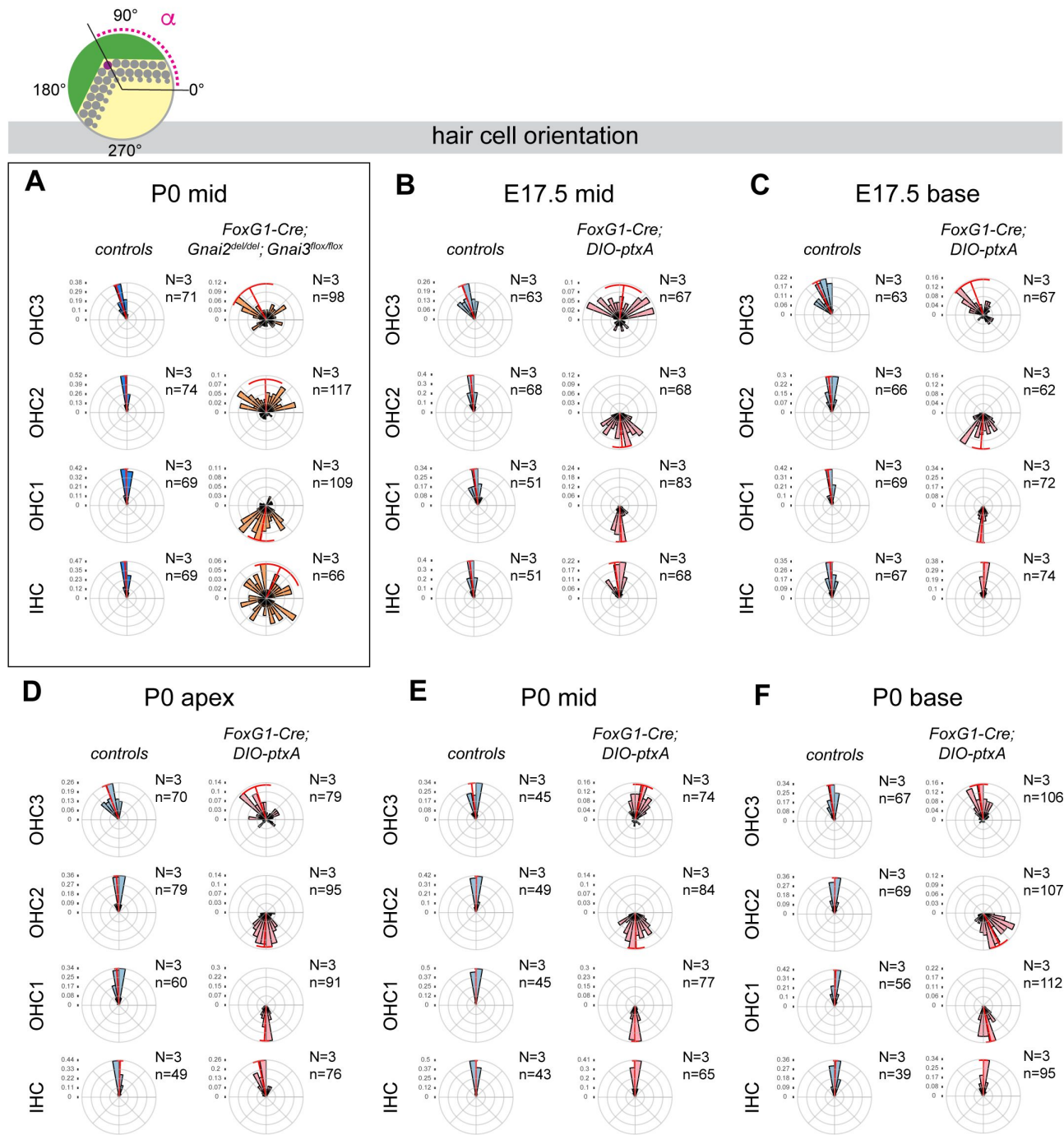


Figure 8 Supplement 1.

Loss of GNAI2 and GNAI3 recapitulates hair cell orientation defects observed in *ptxA* mutants.

A) P0 mid-cochlea histograms that complement P0 apex and P0 base histograms in **Fig. 8C-D**. **B-F)** Histograms including littermate controls for *FoxG1-Cre; DIO-ptxA* mutants in **Fig. 8** (B-C, D, F), and P0 mid-cochlear position for *FoxG1-Cre; DIO-ptxA* mutants and controls (E). Note that all mutant histograms except at the P0 cochlear mid (A, E) are repeated from **Fig. 8**. Circular histograms show HC orientation (α) based on the position of the basal body (purple dot in top diagram) at the stage and cochlear position indicated. 0° is towards the cochlear base and 90° is lateral/abnormal. Only HCs with an eccentricity above 0.25 are represented (see **Fig. 7**). Histograms show frequency distribution (10° bins) and red radial lines and arcs respectively indicate the circular mean and the circular mean deviation. *n*, HC number in N=3-4 animals. Controls for *FoxG1-Cre; Gnai2^{del/del}; Gnai3^{flox/flox}* are *Gnai2^{del/+}; Gnai3^{flox/+}*, *Gnai2^{del/+}; Gnai3^{flox/flox}*, *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/+}* and *FoxG1-Cre; Gnai2^{del/+}; Gnai3^{flox/flox}*. Controls for *FoxG1-Cre; DIO-ptxA* are *DIO-ptxA* heterozygotes. Detailed cohorts and results can be found in the Source Data file.

Gene or model	Official name	Name in the study	MGI #	Reference	Allele description	Genetic background	Genetic background in the study	% surviving mutants at weaning age	
<i>Gnai1</i>	<i>Gnai1^{tm1Lbi}</i>	<i>Gnai1^{neo}</i>		Jiang et al, 2002 PMID: 36092739	neo cassette inserted in exon 3		129S1/SvImJ; C57BL/6J mix	from het x het (expected 25%)	from hom x het (expected 50%)
								16.4% (12 of 73 animals)	45.8% (49 of 107 animals)
<i>Gnai2</i>	<i>Gnai2^{em1Btar}</i>	<i>Gnai2^{del}</i>		this study	CRISPR/Cas9 deletion of exons 2-3-4	C57BL/6J	C57BL/6J	9.9% (15 of 152 animals)	9.6% (5 of 52 animals)
								19.7% (12 of 61 animals)	46.7% (50 of 107 animals)
<i>Gnai3</i>	<i>Gnai3^{tm1Lbi}</i>	<i>Gnai3^{neo}</i>		Jiang et al, 2002 PMID: 36092739	part of intron 5 and exon 6 replaced with a neo cassette		129S1/SvImJ; C57BL/6J mix	30% (12 of 40 animals)	42% (63 of 150 animals)
<i>Gnao1</i>	<i>Gnao1^{tm1Lbi}</i>	<i>Gnao1^{neo}</i>	2152685	Jiang et al, 1998 PMID: 9501252	neo cassette inserted in exon 6	129S1/SvImJ	129S1/SvImJ	from het x het (expected 25%)	
								6.7% (3 animals out of 45)	
<i>Gnai1; Gnai2</i>	<i>Gnai1^{tm1Lbi}; Gnai2^{em1Btar}</i>	<i>Gnai1^{neo}; Gnai2^{del}</i>	from 4456727	KOMP	floxed exon 3 - derived from <i>Gnao1^{tm1a}</i> (EUCOMM)Hmgv via FLP-based removal of LacZneo cassette	C57BL/6N	C57BL/6N	from <i>Atoh1-Cre</i> ; het x het (expected 12.5% cKO)	from <i>Atoh1-Cre</i> ; hom x het (expected 25% cKO)
								40% (10 <i>Atoh1-Cre</i> ; flox hom of 25 animals)	30.7% (4 <i>Atoh1-Cre</i> ; flox hom of 13 animals)
<i>Gnai1; Gnai3</i>	<i>Gnai1^{tm1Lbi}; Gnai3^{tm1Lbi}</i>	<i>Gnai1^{neo}; Gnai3^{neo}</i>	5560183	Jiang et al, 2002 PMID: 36092739		129S1/SvImJ	129S1/SvImJ	from het; het x het; het (expected 6.25%)	from hom; het x hom; het (expected 25% dKO)
								5.8% (7 of 121 animals)	6.9% (2 of 29 animals)
<i>FoxG1-Cre; Gnai2; Gnai3</i>		<i>Gnai2^{del}; Gnai3^{flox}</i>		this study			129S1/SvImJ; C57BL/6J.FVB/NJ mix	second generation crosses	first generation crosses
								0% (0 out of 149 animals)	0.3% (1 out of 333 animals)
<i>ptxA</i>	<i>Gt(ROSA)26Sor^{em1(ptxA)Cgh}</i>	<i>LSL-ptxA.myc</i>	3776029	Regard et al, 2007 PMID: 17992256	<i>loxP-stop-loxP</i> cassette before <i>ptxA.myc</i>	C57BL/6:129SvJ			
	<i>Gt(ROSA)26Sor^{em1(ptxA)Btar}</i>	<i>LSL-myc;ptxA</i>	6163665	Tarchini et al, 2016 PMID: 27660326	<i>loxP-stop-loxP</i> cassette before <i>myc;ptxA</i>	C57BL/6J	C57BL/6J		
		<i>DIO-ptxA</i>		this study	double inverted <i>lox</i> sites flanking <i>ptxA</i>	C57BL/6J	C57BL/6J		
<i>Atoh1-Cre</i>	<i>Tg(Atoh1-cre)1Bfri</i>	<i>Atoh1-Cre</i>	3775845	Matei et al, 2005 PMID: 16145671	randomly inserted transgene with <i>Atoh1</i> enhancer	(C57BL/6 x CBA)F1	C57BL/6		
<i>FoxG1-Cre</i>	<i>Foxg1^{tm1(cre)Skn}</i>	<i>FoxG1-Cre</i>	1932522	Hebert et al, 2000 PMID: 10837119	Cre knock-in in the <i>FoxG1</i> gene	129P2/OlaHsd-HprtB-m3	129S1/SvImJ; C57BL/6J mix		

Supplementary Table 1.

Mouse strain details.

Strain		Primer sequence	Bands size (bp)	
<i>Gnao1^{flox}</i>	forward	5' - TGGGTTTGATCCACAAGAGCGG - 3'		
	forward	5' - AGACGTGAAGCAGTACAAGC - 3'		
	reverse	5' - TCTCCTCAGTAGGGAAGATGGC - 3'		
			wt: 938; flox: 1119	wt: 387; flox: 421
<i>Gnao1^{neo}</i>	forward	5' - TGGTGACCCATTCTGACTTC - 3'		
	forward	5' - TCGCCTTCTATCGCCTTCTTGACG - 3'		
	reverse	5' - TTGGCAGAGAAAGCCCAAAG - 3'		
			wt: 836	neo: 461
<i>Gnai1^{neo}</i>	forward	5' - TCTAGGTGCTGGGGAATCTG - 3'		
	reverse	5' - GAGACGTGCTACTTCCATTTGTC - 3'		
	reverse	5' - TGAGCTTGTAGGCACAGAGG - 3'		
			wt: 1004	neo: 580
<i>Gnai2^{del}</i>	forward	5' -GCACCATCGTCAAGCAGATG - 3'		
	forward	5' - ATGGGTGGTTGTGAGCCATC - 3'		
	reverse	5' - GAGCTGGTATTCTCGTGAGC - 3'		
			wt: 728	wt: 1195; neo: 515
<i>Gnai3^{neo}</i>	forward	5' - CTTCTATCGCCTTCTTGACG - 3'		
	forward	5' -ACTCTGCTGACTTTGGCACC - 3'		
	reverse	5' - CTTTCATGCATTGCGTTCTG - 3'		
			wt: 762	neo: 450
<i>Gnai3^{flox}</i>	forward	5' - ATGAGGACGGCTATTCAAG - 3'		
	reverse	5' - CATCCTACTTCACAACTGC - 3'		XhoI digestion
			wt: 700; flox: 747	wt: 700; flox: 342 + 404
<i>LSL-myc:ptxA</i>	forward	5' - AAAGTCGCTCTGAGTTGTTAT - 3'		
	reverse	5' - GCGAAGAGTTTGTCTCAACC - 3'		
	reverse	5' - GGAGCGGGAGAAATGGATATG - 3'		
			wt: 603	ptxA: 250
<i>DIO-ptxA</i>	forward	5' - GCACTTGCTCTCCCAAAGTC - 3'		
	reverse	5' - GGAGCGGGAGAAATGGATATG - 3'		
	reverse	5' - CGTCATTGACGTCAATAGGG - 3'		
			wt: 617	ptxA: 677
<i>Atoh1-Cre</i>	forward	5' - AGAGCGGCTGACAATAGAGG - 3'		
	reverse	5' - TTTCCATGAGTGAACGAACC - 3'		
			wt: no band	cre: 850
<i>FoxG1-Cre</i>	forward	5' - GCGACAAGAAGAACGGCAAG - 3'		
	forward	5' - GCAAGAACCTGATGGACATG - 3'		
	reverse	5' - AAGCACTTGTTGAGGGACAG - 3'		
	reverse	5' - TTTCCATGAGTGAACGAACC - 3'		
			wt: 202	cre: 381

Supplementary Table 2.

Genotyping strategies.

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Reviewer #1 (Public Review):

A subclass of inhibitory heterotrimeric guanine nucleotide-binding protein subunits, GNAI, has been implicated in sensory hair cell formation, namely the establishment of hair bundle (stereocilia) orientation and staircase formation. However, the former role of hair bundle orientation has only been demonstrated in mutants expressing pertussis toxin, which blocks all GNAI subunits, but not in mutants with a single knockout of any of the *Gnai* genes, suggesting that there is a redundancy among various GNAI proteins in this role. Using various conditional mutants, the authors concluded that GNAI3 is the primary GNAI proteins required for hair bundle morphogenesis, whereas hair bundle orientation requires both GNAI2 and GNAI3.

Strength

Various compound mutants were generated to decipher the contribution of individual GNAI1, GNAI2, GNAI3 and GNAIO in the establishment of hair bundle orientation and morphogenesis. The study is thorough with detailed quantification of hair bundle orientation and morphogenesis, as well as auditory functions.

Weakness

While the hair bundle orientation phenotype in the *Foxg1-cre; Gnai2^{-/-}; Gnai3 lox/lox* (double mutants) appear more severe than those observed in *Ptx cKO* mutants, it may be an oversimplification to attribute the differences to more GNAI function in the *Ptx cko* mutants. The phenotypes between the double mutants and *Ptx cko* mutants appear qualitatively different. For example, assuming the milder phenotypes in the *Ptx cKO* is due to incomplete loss of GNAI function, one would expect the *Ptx* phenotype would be reproducible by some combination of compound mutants among various *Gnai* genes. Such information was not provided. Furthermore, of all the double mutant specimens analyzed for hair bundle orientation (Fig. 8), the hair bundle/kinocilium position started out normally in the lateral quadrant at E17.5 but failed to be maintained by P0. This does not appear to be the case for *Ptx cKO*, in which all affected hair cells showed inverted orientation by E17.5. It is not clear whether this is the end-stage of bundle orientation in *Ptx cKO*, and the kinocilium position started out normal, similar to the double mutants before the age of analysis at E17.5. Understanding these differences may reveal specific requirements of individual GNAI subunits or other factors are being affected in the *Ptx* mutants.

Reviewer #2 (Public Review):

Jarysta and colleagues set out to define how similar GNAI/O family members contribute to the shape and orientation of stereocilia bundles on auditory hair cells. Previous work demonstrated that loss of particular GNAI proteins, or inhibition of GNAs by pertussis toxin, caused several defects in hair bundle morphogenesis, but open questions remained which the authors sought to address. Some of these questions include whether all phenotypes resulting from expression of pertussis toxin stemmed from GNAI inhibition; which GNAI family members are most critical for directing bundle development; whether GNAI proteins are needed for basal body movements that contribute to bundle patterning. These questions are important for understanding how tissue is patterned in response to planar cell polarity cues.

To address questions related to the GNAI family in auditory hair cell development, the authors assembled an impressive and nearly comprehensive collection of mouse models. This approach allowed for each *Gnai* and *Gnao* gene to be knocked out individually or in combination with each other. Notably, a new floxed allele was generated for *Gnai3* because

loss of this gene in combination with *Gnai2* deletion was known to be embryonic lethal. Besides these lines, a new knockin mouse was made to conditionally express untagged pertussis toxin following cre induction from a strong promoter. The breadth and complexity involved in generating and collecting these strains makes this study unique, and likely the authoritative last word on which GNAI proteins are needed for which aspect of auditory hair bundle development.

Appropriate methods were employed by the authors to characterize auditory hair bundle morphology in each mouse line. Conclusions were carefully drawn from the data and largely based on excellent quantitative analysis. The main conclusions are that GNAI3 has the largest effect on hair bundle development. GNAI2 can compensate for GNAI3 loss in early development but incompletely in late development. The *Gnai2 Gnai3* double mutant recapitulates nearly all the phenotypic effects associated with pertussis toxin expression and also reveals a role for GNAs in early movement of the basal body. Although these results are not entirely unexpected based on earlier reports, the current results both uncover new functions and put putative functions on more solid ground.

Based on this study, loss of GNAI1 and GNAO show a slight shortening of the tallest row of stereocilia but no other significant changes to bundle shape. Antibody staining shows no change in GNAI localization in the *Gnai1* knockout, suggesting that little to no protein is found in hair cells. One caveat to this interpretation is that the antibody, while proposed to cross-react with GNAI1, is not clearly shown to immunolabel GNAI1. More than anything, this reservation mostly serves to illustrate how challenging it is to nail down every last detail. In turn, the comprehensive nature of the current study seems all the more impressive.